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**La Evolución del Pie de las Aves: una  
Mirada desde el Desarrollo**

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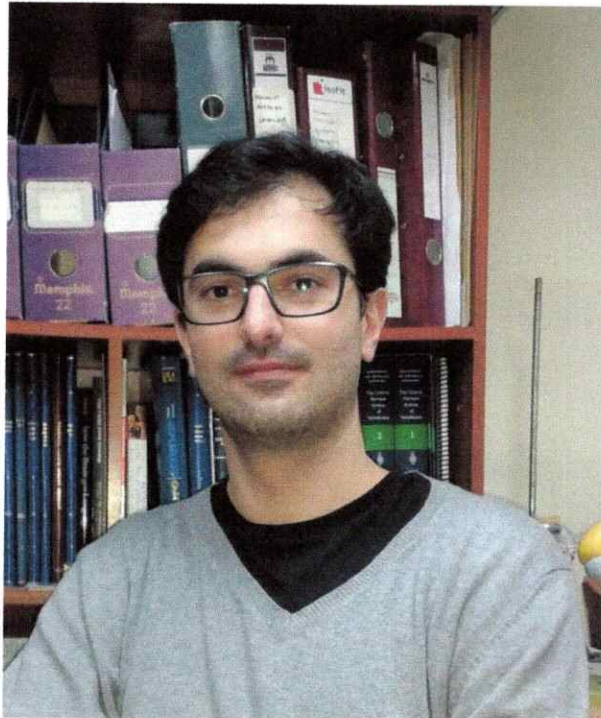
## **La Evolución del Pie de las Aves: una Mirada desde el Desarrollo**

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Santiago, 2014

## RESUMEN BIOGRAFICO



Joao Francisco Botelho nació en el litoral de Santa Catarina, Brasil, el año de 1979. Tuvo una infancia tranquila entre la playa y el bosque tropical. A los diecisiete años ingresó a la carrera de Ciencias Biológicas de la Universidade Federal de Santa Catarina. En 2005 ingresó al Magíster de Filosofía en la misma universidad. En 2008 entró al programa de Doctorado en Ciéncial en la Universidad de Chile. Sus intereses incluyen la historia y filosofía de la biología, la relación entre evolución y desarrollo, y la evolución de las aves.

*The fore-limbs, for instance, which served as legs in the parent-species, may become, by a long course of modification, adapted in one descendant to act as hands, in another as paddles, in another as wings; and on the above two principles — namely of each successive modification supervening at a rather late age, and being inherited at a corresponding late age—the fore-limbs in the embryos of the several descendants of the parent species will still resemble each other closely, for they will not have been modified.*

Charles Darwin, 1859

*Thus we discern, in the main differential character of the by-fossil-remains-oldest-known feathered Vertebrate, a retention of a structure embryonal and transitory in the modern representatives of the class, and closer adhesion to the general vertebrate type.*

Richard Owen, 1864

*...if the whole hind quarters, from the ilium to the toes, of a half-hatched chicken could be suddenly enlarged, ossified, and fossilized as they are, they would furnish us with the last step of the transition between Birds and Reptiles; for there would be nothing in their characters to prevent us referring them to the dinosauria.*

Thomas Huxley, 1869

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## RESUMÉN

### La Evolución del Pie de las Aves: una Mirada desde el Desarrollo

El actual conocimiento de la sistemática y del registro fósil muestra como mucho de los rasgos del esqueleto de las aves son diferentes a los de sus ancestros. En el esqueleto de la pata, uno de los principales cambios es la re-orientación de los dígitos. Terópodos basales presentaban cuatro dígitos orientados paralelamente. Las aves re-orientaron el hallux a una posición oponible, permitiendo a la pata agarrar. En algunos grupos de aves vivientes otras orientaciones evolucionaron secundariamente. Las re-orientaciones de los dígitos ocurren durante el desarrollo del esqueleto. En esta tesis investigamos la relación entre los mecanismos que controlan la re-orientación de los dígitos durante el desarrollo y la evolución de estos rasgos. Primero, mostramos cómo un retraso en la diferenciación del metatarso 1 permite a la actividad muscular embrionaria reorientar el hallux a una posición oponible. En seguida, mostramos cómo una asimetría en la musculatura de la pata es responsable del desarrollo de un segundo dígito oponible en aves Psittaciformes. Por último, mostramos cómo la evolución de innovaciones en la orientación de los dígitos en todas las aves modernas depende de heterocronías en el desarrollo del esqueleto relacionadas a la altricialidad. Los datos llaman la atención hacia la influencia de dos mecanismos ontogenéticos en la evolución del pie de las aves: heterocronías en los tiempos de diferenciación del esqueleto y la influencia de la actividad muscular embrionaria sobre la morfología del esqueleto.

## ABSTRACT

### The Evolution of Bird's Foot: a Developmental Approach

Current knowledge of systematics and the fossil record give a good picture of how many of the features of the avian skeleton changed from their ancestors. In the foot skeleton, one of the main changes is the re-orientation of the digits. Basal theropods have four digits oriented in parallel. Birds re-orient the hallux to an opposable position, allowing the foot to grasp. Some groups of living birds secondarily evolved other orientations. The re-orientation of the digits occurs during skeletal development. In this thesis we investigate the relationship between the mechanisms controlling the re-orientation of the digits during development and the evolution of those features. First, we show how a delayed differentiation of the metatarsal 1 allows embryonic muscle activity to re-orient hallux to an opposable position. Then, we show how an asymmetry in the foot musculature is responsible for the development of a second opposable digit in Psittaciformes birds. Finally, we show that the evolution of innovations in the orientation of the digits in all modern birds depends on heterochronies in skeletal development related to altriciality. The data draw attention to the influence of two ontogenetic mechanisms in the evolution of the avian foot: heterochronies altering the times of differentiation of the skeleton and the influence of muscle activity on embryonic skeletal morphology.



## INTRODUCCIÓN

### 1. Filogenia, Ontogenia y Morfología

Las citas de los tres grandes naturalistas de la Inglaterra victoriana en el epígrafe de esta tesis - Darwin, Owen y Huxley -, reflejan la confusa relación que existía durante la segunda mitad del siglo XIX entre las ideas de transformación individual del embrión y de transformación trans-generacional del linaje. La comparación entre adultos, embriones y fósiles de diferentes especies indicaba la existencia de algún tipo de relación entre estos dos procesos de transformación histórica de los seres vivos. Pero en un periodo en que los propios conceptos de herencia, desarrollo, evolución, ontogenia y filogenia estaban siendo formulados, el entendimiento de esta relación permanecía obscuro. El caso de las aves es emblemático. Sus embriones, cuando comparados a embriones de otros tetrápodos, revelaban similitudes insospechadas en la morfología de los adultos. El ala, la cola, el pie y el cráneo

de las aves – tan distintos a los de cualquier otro grupo de tetrápodos – develaban sus afinidades con una simple comparación entre los embriones. Como en muchos otros casos, era evidente que el esqueleto de las aves era al principio similar al de los otros tetrápodos y durante el desarrollo embrionario se modificaba radicalmente.

Después de la consolidación de la teoría de la evolución, en las últimas décadas del siglo XIX, este tipo de observación llevó a las concepciones recapitulacionistas de la relación entre la ontogenia y la filogenia (Gould 1977). Es decir, se creía que la serie de transformaciones evolutivas de una linaje sería recapitulada en cada serie de transformaciones embrionarias de un individuo. La ontogenia del esqueleto de las aves, por lo tanto, recapitularía su historia evolutiva, pasando por la morfología de sus ancestros anfibios y reptiles, hasta llegar a su forma específica de ave. Esta ley biogenética, como era llamada por Haeckel, también implicaba un modo de transformación evolutiva (Haeckel 1866). La evolución ocurriría por la adición de modificaciones producidas por la selección natural al final de la ontogénesis. Por lo tanto, las variaciones y adaptaciones propias de las patas de cada grupo serían fruto de la acumulación de transformaciones de la morfología ancestral durante los períodos tardíos del desarrollo, conservándose al principio de éste una morfología común.

La ley biogenética ha sido olvidada con la maduración de la biología celular y la biología del desarrollo, en el inicio del siglo XX (Garstang 1922). Sin embargo, junto con el recapitulacionismo, ha sido abandonado gran parte del interés por el papel del desarrollo en la evolución. Durante la mayor parte del siglo XX, el estudio de la evolución ha dejado de lado la morfología y la ontogenia, y se ha centrado en sus aspectos genéticos y poblacionales (Hamburger 1980). En este contexto, el hecho de que el esqueleto de los embriones de las aves se parezca al esqueleto de reptiles adultos no despertaba el interés de los evolucionistas.

Sin embargo, en las últimas décadas hubo un renacimiento del interés por la influencia del desarrollo en las transformaciones evolutivas (Müller 1991, Raff 1995, Hall 1999, Arthur 2004). Las nuevas posibilidades de investigación de la biología molecular, biología celular y biología del desarrollo, integrados a la morfología, paleontología y sistemática hicieron de la Biología Evolutiva del Desarrollo unos de los campos más prolíficos de la Biología Evolutiva contemporánea.

El objetivo general de esta tesis puede ser resumido como un intento de examinar el problema levantado por las afirmaciones de Owen, Darwin y Huxley, en el siglo XIX, desde las posibilidades abiertas actualmente por la Biología Evolutiva del Desarrollo. Buscamos entender como las transformaciones ontogenéticas de diferentes aspectos de la morfología del



esqueleto de las aves están relacionadas con su evolución. Para esto, integramos el conocimiento de diferentes áreas de la biología experimental y de la historia natural. Los resultados están presentados en la forma de tres artículos. Cada artículo está precedido por su propia introducción. Por lo tanto, esta introducción general se limitará a una breve presentación de tres elementos necesarios para la comprensión del problema: las relaciones filogenéticas de las aves, la morfología del esqueleto de la pata de las aves y su ontogenia.

### 1.1 La Filogenia de las Aves

La investigación del desarrollo es de poca ayuda para el entendimiento de la evolución si no se conoce la secuencia de transiciones morfológicas que produjeron los diferentes linajes. Si bien es cierto que no es viable conocer cada paso en la historia de cambios de un linaje, una mirada comparativa entre los grupos vivientes y fósiles permite inferir cuales transformaciones morfológicas originaron los rasgos de nuestro interés; y una mirada comparativa adecuada depende del entendimiento adecuado de las relaciones genealógicas entre los grupos. Por lo tanto, en el contexto la presente investigación sobre la evolución de la pata de las aves, es menester

conocer como las aves están relacionadas genealógicamente entre sí y con los demás vertebrados.

Las aves son vertebrados bastante inusuales. Poseen el cuerpo cubierto de plumas; un pico córneo sin dientes, alas en vez manos, un pigostilo en vez de cola. No sorprende que sus afinidades con otros vertebrados hayan permanecido elusivas por mucho tiempo. Carl Linnaeus, en su *Systema Naturae*, agrupó las aves en su propia Clase. Para Jean Baptiste Lamarck las aves derivaban de peces voladores. Sir Richard Owen las clasificó junto a los Pterodáctilos. Sin embargo, desde que la formulación de hipótesis filogenéticas pasó al centro de las ciencias naturales en el período post-darwinista, las aves fueron prontamente relacionadas a los reptiles (Bowler 1996). Esta hipótesis, así como la propia Teoría de la Evolución, fue favorecida por el descubrimiento de los primeros ejemplares de *Archaeopteryx*, en 1861, poco después de que Charles Darwin publicara la primera edición de *On the Origin of the Species* (Darwin 1859). *Archaeopteryx* presenta claramente rasgos reptilianos. Después de su descubrimiento, la pregunta sobre las relaciones de las Aves cambió de foco. Era evidente que las aves eran reptiles. La nueva pregunta era ¿cuál grupo específico de reptiles, fósil o viviente, estaría más estrechamente relacionado a las aves?

Thomas Huxley (1870) fue el primero en defender la relación entre las aves y los pocos dinosaurios fósiles conocidos hasta entonces, pero su interpretación no prosperó en el siglo XX. La interpretación hegemónica, hasta los años 1980, era de que las aves habían evolucionado de un grupo hipotético de arcosaurios basales, no siendo cercanamente emparentadas o derivadas de los otros grandes grupos Archosauria, tales como Pterosauria, Dinosauria o Crocodylia (Broom, 1913, Heilmann, 1926, Simpson, 1946).

Sin embargo, desde que Ostrom (Ostrom 1973, Ostrom 1975) rescató la hipótesis de Huxley, la interpretación de que las aves derivan de dinosaurios terópodos alcanzó el consenso entre los sistemáticos (Figura 1.1). Dos factores ayudaron a consolidar la hipótesis terópodo-aves. Primero, la utilización de la metodología cladística permitió un análisis más preciso y falsable de la filogenia. Diversos análisis independientes indicaron que aves y terópodos maniraptores forman un grupo monofilético (Gauthier 1986, Benton and Clark 1988, Sereno 1991). Segundo, el descubrimiento de una gran variedad de dinosaurios terópodos y aves mesozoicas fósiles en los últimos 30 años, especialmente en China, España, Madagascar y Argentina, cerró muchas de las lagunas morfológicas entre terópodos y aves.

Los dinosaurios están divididos en dos grandes grupos: Ornithischios y Saurischios. Este último, a su vez, se divide en otros dos:

Sauropodomorpha y Theropoda (Figura 1). Las aves pertenecen a una rama de los terópodos llamada Maniraptores, que incluyen formas originalmente herbívoras; de metatarsos alargados y cerebros relativamente grandes, como Ornithomimosauros, Oviraptorosauros, Therizinosauros, Alvarezsauridos y Deinonychosaurios (Figura 1). Dentro de Maniraptora, las Aves son agrupados con Deinonychosauria en un clado llamado de Eumaniraptora (Turner et al. 2012):

Son consideradas Aves (o Avialae) todas las especies que comparten un ancestro común más reciente con las aves modernas que con *Archaeopteryx*. Varios Eumaniraptores descubiertos recientemente son muy similares a *Archeopteryx*. Algunos, como *Aurornis* y *Xiaotingia*, han sido agrupados inmediatamente afuera de Aves, mientras que otros, como *Jeholornis* and *Jixiangornis*, han sido considerados más derivados que *Archaeopteryx*, y por lo tanto, aves (Li et al. 2010, Xu et al. 2011, Godéffroit et al. 2013). Es dentro del linaje de las aves, en el clado Pygostila (en referencia al pigostilo originado por la fusión de vertebras de la cola) que las aves adquieren su máxima diversificación. En la base de Pygostila están fósiles como *Sapeornis* y *Confuciusornis*, aves mesozóicas ya con algunas de las características comunes en el esqueleto las aves modernas (Chiappe et al. 1999, Zhou and Zhang 2003, Zhang et al. 2009) y también la primera gran radiación de las aves en el clado

Enantiornithes. Fósiles de estas aves han sido encontrados en todos los continentes y en diferentes paleoecosistemas. Diversas de las transformaciones morfológicas presentes en las aves vivientes ocurren paralelamente en Enantiornithes, como la reducción del número de falanges y la pérdida de los dientes.





El grupo hermano de Enantiornithes es llamado Ornithothoraces e incluye a las aves modernas. Diversos Ornithothoraces basales han sido descritos en las últimas décadas, como *Patagopteryx* y *Vorona*. Sus patas se caracterizan por la completa fusión de los metatarsales, fusión de los tarsales y torsión del metatarsus 1. *Hesperornis* y *Ichthyornis*, las únicas aves fósiles conocidas en el siglo XIX – además de *Archaeopteryx* –, son el grupo hermano de Neornithes, el grupo de las aves vivientes. Neornithes comprende cerca de 10.000 especies. Sus patas se caracterizan por un hipotarso complejo y variaciones en la orientación de los dígitos. La primera divergencia es representada por las Paleognathas (avestruces, kiwis y afines) y Neognathas. Dentro de este último, el primer clado a divergir es Galloanserae (patos, pollos y afines). El resto de la aves pertenece al clado Neoaves. Aunque varios grandes grupos dentro de Neoaves están bien establecidos (ver capítulo 4), las relaciones entre ellos permanecen inciertas.

## 1.2 La Morfología de la pierna de las Aves

La organización básica del esqueleto del miembro posterior de los tetrápodos es extremadamente conservada. En la región proximal, el estilópodo, hay un único elemento – el fémur. Hacia distal, en el zeugópodo, hay dos elementos paralelos – la tibia y la fíbula. En el extremo distal, en el autópodo,

están los dígitos, que originalmente son cinco, pero esta cantidad es frecuentemente reducida. Entre el zeugópodo y el autómodo se encuentran un número variable de elementos globulares – los tarsales. Esta organización básica, fácilmente reconocible en dinosaurios basales, ha sido bastante transformada durante la evolución de las aves, originando una morfología singular (Figura 1.2).

Al ser comparado con la condición ancestral, el fémur es el elemento evolutivamente más conservado del esqueleto de la pierna de las aves. Los fósiles muestran que la mayoría de las variaciones ocurrieron en las trócleas proximales y están asociadas a cambios en el modo de locomoción (revisado en Hutchinson 2001). En la región del zeugópodo la morfología del esqueleto es mucho más divergente. La condición ancestral de la tibia y la fíbula de las aves es la presencia de elementos de tamaños similares, ambos articuladas a los huesos tarsales libres. Esta condición está presente también en Aves basales como *Archaeopteryx*. Sin embargo, dentro de Pygostilia, la fíbula está reducida, estrechándose hacia su extremo distal en forma de aguja. Es notablemente más corta que la tibia y ya no participa de la articulación del tobillo.

El pie de las aves es extremadamente derivado. La articulación del tobillo ocurre entre los tarsales proximales y distales. En las aves vivientes no

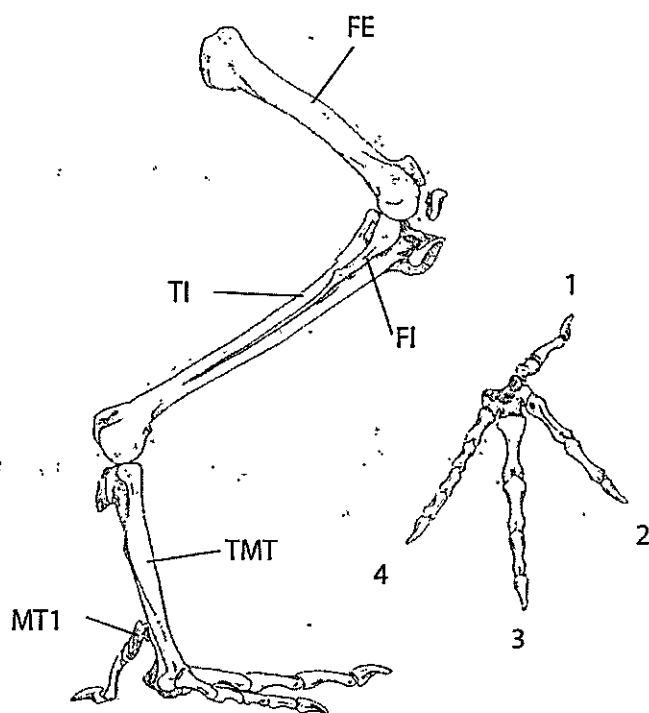


Figure 1.2. El esqueleto de la pata del pollo doméstico (*Gallus gallus*) en vista lateral y distal. FE: fémur; TI: tibia; FI: fibula; TMT: tarsometarsus; MT1: metatarsal 1; Modificado de Raikow, 1986

hay huesos tarsales libres cuando adultas. Tres elementos proximales (el fibulare el tibiale y el proceso acendiente) se fusionan a la tibia formando el tibiotarso. El único tarsal distal también se fusiona, pero a los metatarsales. La mayoría de las aves modernas presentan cuatro dígitos en los pies. Los metatarsales de los dedos 2, 3 y 4 están fusionados en las aves adultas a lo largo de su eje próximo-distal, generando el tarsometatarso. El arreglo de los dedos varía dentro de Neoaves, siendo la anisodactilia la condición más común – el halux revertido y los otros tres dedos orientados hacia adelante.

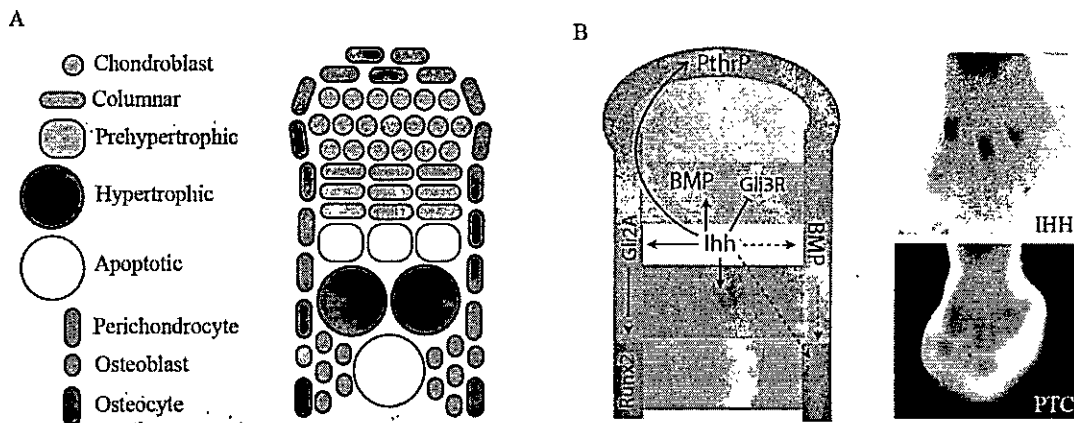
### 1.3 La Ontogenia del Miembro Posterior de Las Aves

Las características singulares del esqueleto de las aves son adquiridas durante el desarrollo embrionario. El conocimiento sobre las interacciones celulares y moleculares que ocurren durante el desarrollo de la pata y su sistema esquelético ha avanzado mucho en las últimas décadas, permitiendo nuevas aproximaciones hacia el problema de su evolución.

El esqueleto de la pata se origina por osificación endocondral. Inicialmente, células no diferenciadas originadas en el mesodermo lateral forman condensaciones mesenquimales en el centro del brote del miembro. Las células de las condensaciones mesenquimales se diferencian en condrocitos y originan un "molde" de cartílago en miniatura del futuro hueso (Figura 1.3C). El proceso de diferenciación de mesénquima a condrocitos es llamado *condrogénesis primaria* y es caracterizado por cambios en la forma celular y por la secreción de proteínas de la matriz celular. El patrón de condrogénesis primaria en los miembros es bastante conservado entre los tetrápodos y esto, en parte, explica por qué los esqueletos de embriones tempranos de diferentes especies son parecidos. En el caso de la pata, la primera condensación ocurre en la porción proximal y origina el estilópodo (fémur); posteriormente la condensación del estilópodo se bifurca en la

extremidad distal y origina el zeugópodo (fíbula y tibia); por último, se forman los elementos del autópodio (tarsales, metatarsales y falanges) (Figura 2A). La regulación del patrón de condrogénesis primaria ha sido utilizada como sistema modelo desde la embriología experimental y muchos de los eventos moleculares involucrados en ésta han sido caracterizados (para una revisión ver Towers y Tickle (2009)).

La condrogénesis primaria es seguida por una etapa de *condrogénesis secundaria*, cuando los condrocitos proliferan y se diferencian, dando forma al elemento. Indian hedgehog (Ihh) es el principal factor caracterizado en la condrogénesis secundaria, promoviendo tanto la proliferación de los condrocitos (Vortkamp et al. 1996, Kronenberg et al. 1997, Karp et al. 2000, Minina et al. 2001), como su diferenciación (Hartmann and Tabin 2000, Minina et al. 2002, Amizuka et al. 2004, Hu et al. 2005). El efecto de Ihh es mediado por efectores de esta vía, como Ptc y Gli1 y Gli3 y también por la interacción con otras vías, especialmente con PTHrP (Hilton et al. 2005, Koziel et al. 2005) (Figura 1.3B). Al final de la condrogénesis, los condrocitos hipertróficos mueren por apoptosis, dejando la matrix extracelular para la invasión de osteoblastos.



**Figura 1.4.** (A) Diagrama de una de la epífisis mostrando la distribución de los diferentes tipos de condrocitos durante la osificación endocondral ; (B) Diagrama mostrando el papel central de IHH en el proceso de osificación endocondral; (C) Hibridización *in situ* mostrando la expresión de *Ihh* y *Ptc* en los metatarsales de un embrión de pollo en HH30.

El patrón de crecimiento durante la condrogénesis secundaria y la osificación determinan la forma básica de cada elemento del esqueleto. Sin embargo, la morfología del esqueleto depende también de la interacción con los músculos y los tendones durante el desarrollo embrionario. Los precursores de los músculos y tendones se originan en los somitos y migran tempranamente hacia el brote del miembro. Una vez en la pata, se diferencian en grandes masas de músculos y tendones que se subdividen hasta formar las estructuras individuales.

El desarrollo del esqueleto quizá ofrece la evidencia más contundente de la relación entre dinosaurios y aves. Coincidencia o no, tanto la investigación del papel de la ontogenia como mecanismo transformador de la filogenia como la hipótesis de que las aves están emparentadas a los dinosaurios estuvieron eclipsadas juntas durante grande parte del siglo XX. Investigar cómo el esqueleto de la pata de las aves desarrolla sus características singulares ofrece la posibilidad no solamente de entender su evolución, sino que también la relación entre la evolución y el desarrollo.

Esta tesis aborda los mecanismos de desarrollo de las reorientaciones de los dígitos de las aves y sus consecuencias evolutivas. En el primer capítulo se examina el desarrollo del halux oponible; el segundo capítulo se trata de la adquisición de un segundo dígito oponible en ciertas aves modernas; y finalmente, el tercer capítulo investiga la relación entre los modos de desarrollo y las variaciones en la morfología del pie de las aves modernas.



## The Development of the Opposable Hallux in the Bird Foot and its Evolutionary Implications

**Abstract:** Most extant birds have an opposable hallux that allows the feet to grasp, which evolved from the non-opposable hallux of early theropod dinosaurs. Morphological comparisons between avian and non-avian theropods indicate that the acquisition of a retroverted hallux is caused by the displacement of the articulation of metatarsal 1 (Mt1) to the ventral side of metatarsal 2 (Mt2), and by the twist of the long axis of Mt1. To understand the developmental mechanisms involved in the evolution of the opposable hallux, we studied the ontogeny of these traits in chicken and quail embryos. The reorientation of the hallux begins with its flexion perpendicular to Mt2 and its displacement to the ventral side of the foot. The twist of Mt1 begins at embryonic day 10 and causes a change from a medial orientation to a fully opposable one. This coincides with the establishment of muscular connectivity and the onset of embryonic motility of the hallux. Embryos paralyzed at HH34 do not fully retrovert the hallux and have a straight Mt1 shaft, phenocopying the morphology of Mt1 of early theropods. Mt1 shows slow cartilage differentiation and late ossification, suggesting a prolonged phase of early plasticity that allows Mt1 to become twisted by the action of muscular activity. These results stress the importance of embryonic muscular activity and rates of ontogenetic processes for understanding the evolution of this major innovation of the avian lineage.

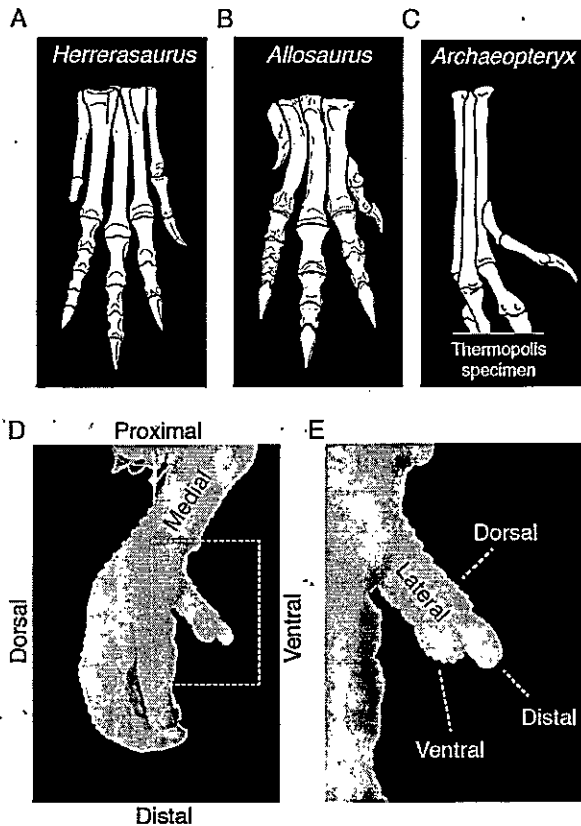
**Keywords:** bird evolution, dinosaurs, foot, opposable hallux.

### 3.1 Introduction

A major morphological modification in the evolution of birds is the acquisition of an opposable hallux (Sereno and Rao 1992). The foot of tetanuran dinosaurs (including birds) has four digits, but digit 1 (D1, the hallux) has lost its proximal articulation to the tarsus. The shape of metatarsal 1 (Mt1) tapers towards its proximal end, presenting a non-synovial articulation to metatarsal 2 (Mt2). In early tetanurans, the foot stood on digits 2, 3 and 4 but D1 was not opposable and was similar to a dewclaw, hanging from Mt2 (figure 1A, B). In early Paraves (Scansoriopterygidae + Dromaeosauridae + Troodontidae + Avialae), D1 became larger and at level with the other digits (figure 1C). Thereafter, during the early evolution of birds, the hallux became opposable to the other digits, allowing the foot to grasp (figure 1D). By this means, the opposable hallux came to be intimately associated with the way of life of many modern birds and its evolution is often discussed in the debate about the cursorial or arboreal origin of birds (Padian and Chiappe-1998).

Modern birds show different grades of hallux opposability, ranging from non-opposable, (*e.g.* Apterygidae or Cariamae) to fully opposable (*e.g.* Galliformes or Passeriformes). This variation has been ascribed to two factors: (i) the position of the articulation between the Mt1 and the medial-to-ventral face of Mt2: the more ventral the joint, the more opposable is the hallux (figure 2H-J, compare boobies and songbirds); and (ii) the torsion of Mt1 along its longitudinal axis (Middleton 2001) (figure 2 A-G). Consequently, in fully opposable halluces, the orientation is inverted and the originally

lateral side of the digit faces towards the medial side of the foot (figure 1E).



**Figure 2.1: Major transformations of Mt1 in the evolution of avian opposable hallux.**

(A) Basal dinosaur exhibit the Mt1 (white arrow) articulated to distal tarsals (*Herrerasaurus*) (Serenó 1993); (B) Mt1 of basal tetanurans had lost its proximal epiphysis and articulated to Mt2 (*Allosaurus*); (C) Basal birds had the Mt1 articulated to the distal Mt2 (*Archaeopteryx*); (D) Most extant birds exhibit the hallux opposed to other toes, like observed in late chicken embryo (HH44); (E) Detail showing hallux inverted orientation.

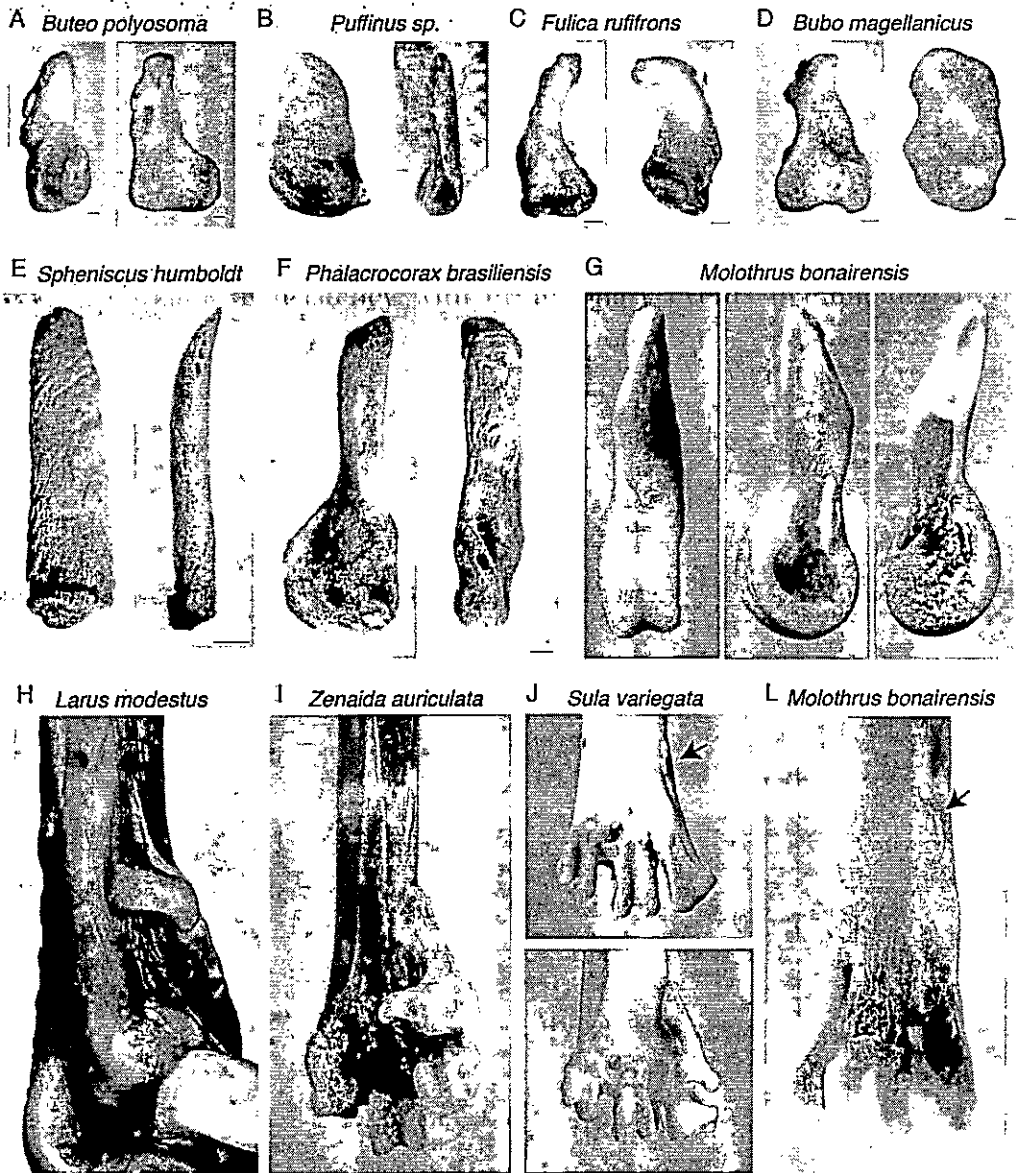
In early tetanurans with an elevated, non-opposable hallux, Mt1 was straight, showing no torsion, and articulated onto the medial surface of Mt2. Historically, *Archaeopteryx* has been thought to have an opposable hallux (Wellnhofer 2009). However, this interpretation was recently challenged by the report of the tenth *Archaeopteryx* specimen (Mayr et al. 2005). While the previously known *Archaeopteryx* fossils did not provide enough information or suggested an opposable orientation of the hallux (Wellnhofer 1974, Thulborn and Hamley 1982), the tenth specimen, in which

both feet are preserved articulated, shows that D1 was not retroverted: Mt1 articulated to the medial surface of Mt2 and its shaft was straight (Mayr et al. 2005, Mayr and Peters 2007, Mayr et al. 2007). Therefore, an opposable hallux evolved in birds more closely related to Neornithes (crown birds) than *Archaeopteryx*.

Beyond these anatomical considerations, the embryonic development of the hallux in the foot of extant birds remains poorly understood. The hallux of early bird embryos is not retroverted, as the cartilaginous precursors of the four toes are initially oriented in the same plane and connected by interdigital tissue (Heilmann 1926). The opposable orientation of the hallux is acquired later in development, when digits are separated and the foot skeleton has begun to ossify. To date, there is no developmental data explicitly addressing the position of the non-synovial articulation or the twisted shape of Mt1.

Here, we studied the development of the hallux in two Galliformes birds – the Japanese quail and the domestic chicken. We determined when and how the digit changes its orientation and the temporal relation of this process with the differentiation of cartilage and the development of the associated musculoskeletal system. Using experimental muscular paralysis, we assessed the role of embryonic muscular activity in the development of the hallux. Our developmental data shows remarkable consistency with evolutionary events documented by the fossil record, thus shedding light on the mechanisms involved in the evolution of hallux opposability. musculoskeletal system. Using experimental muscular paralysis, we assessed the role of embryonic muscular activity in the development of the hallux. Our developmental data

shows remarkable consistency with evolutionary events documented by the fossil record, shedding light on the mechanisms involved in the evolution of hallux opposability.



**Figure 2.2: Variation Mt1 form and fossa metatarsi I position in extant birds** The morphology, in different views, of the Mt1 of (A) Red-backed hawk (*Geranoaetus polyosoma*); (B) a petrel (*Puffinus sp.*); (C) Red-fronted coot (*Fulica rufifrons*); (D) Magellanic horned owl (*Bubo magellanicus*); (E) Humboldt penguin (*Spheniscus humboldti*); (F) Neotropic cormorant (*Phalacrocorax brasiliensis*); (G) Shiny cowbird (*Molothrus bonairensis*); (H) Franklin's gull (*Leucophaeus modestus*) in medial view; (I) Chilean eared dove (*Zenaida auriculata*) in plantar view; (J) Peruvian booby (*Sula variegata*) in ventral view; (L) Shiny cowbird (*Molothrus bonairensis*) in ventral view

## **2.2. Material and Methods**

### **(a) Nomenclature**

We follow an anatomical terminology based on Baumel and Witmer (Baumel et al. 1993) complemented with more specialized literature. The series of chicken and quail development are based on Hamburger and Hamilton (HH) stages (Hamburger and Hamilton 1951).

### **(b) Experimental Animals and Skeleton Collections**

Fertilized Broiler chicken eggs were purchased from a local farm and incubated at 37.5°C and 70% humidity in an incubator with automatic rotating shelves. Fertilized quail eggs were obtained from the colony maintained at the University of Chile and incubated in the same conditions as the chicken eggs. All animal procedures were in accordance with the Chilean legislation and were approved by Institutional Animal Care and Use Committees. All bird adult skeletons belong to the Osteological Collection of the Vertebrate Zoology Laboratory (UCHZV), University of Chile and were photographed with a Canon macroscopic lens.

### **(c) Skeletal Staining**

Complete developmental series for quail and chicken were prepared for cartilage and bone staining. Embryos were fixed in 100%

methanol for 2-3 days at room temperature (RT). For cartilage staining, methanol was replaced by 5:1 ethanol:acetic acid solution with 0,02% 8G Alcian Blue (Sigma-Aldrich) for two days at RT in an orbital shaker. For bone staining, embryos were rehydrated and post-fixed in 10% formalin for 1 hour at 4°C. Following two 10-minute washes with a solution of 0.5% KOH and 50% ethanol, embryos were stained with 0.02% Alizarin Red (Sigma-Aldrich) in 0.5% KOH for 1 hour at RT. Then, embryos were cleared in a sequence of 1:3, 1:1 and 3:1 glycerol:water. For the double staining of both cartilage and bone, Alcian Blue staining was shortened to one day in order to avoid decalcification and formalin post-fixation was omitted. For fluorescent staining of bones, embryos were fixed in 4% of paraformaldehyde solution, washed and incubated for 2 hours in 10µl/ml of Calcein (Sigma-Aldrich). Next they were washed, cleared with Urea 4M and photographed in a Fluorescent Stereoscopic Microscope (Nikon). Paraffin sections were cut at 7µm thick and stained with Safranin and Hematoxylin employing standard histological protocols.

#### (d) Whole Mount Immunofluorescence

Four quail embryos for each stage were prepared for each assay. Embryos were fixed in Dent's Fix (4:1 methanol:DMSO) for 2 hours at RT, dehydrated in 100% methanol and left at -80°C overnight. Before



immunostaining, they were bleached in Dent's Bleaching (4:1:1 methanol:DMSO:H<sub>2</sub>O<sub>2</sub>) for 24h at RT. For anti-collagen immunostaining, embryos were dissected and digested with 2mg/ml of hyaluronidase (Sigma) in PBS for 2 hour at 37°C. Before immunostainings, embryos were rehydrated in PBS 1% triton (PBST) and incubated in primary antibodies for two days at 4°C in an orbital shaker. Primary antibodies were diluted in 2% horse serum, 5% DMSO in PBST at the following concentrations: 1:20 anti-myosin 2 (MF-20, Developmental Studies Hybridoma Bank, DSHB); 1:10 anti-tenascin (M1-B4, DSHB); 1:40 anti-collagen type II (II-II6B3, DSHB); 1:20 anti-collagen type X (X-AC9); and 1:200 anti-hedgehog (shh-160, Santa Cruz Biotechnology). Secondary antibodies anti-mouse and anti-rabbit (Alexa-488 and Alexa-Fluor 594, Jackson ImmunoResearch, PA) were diluted in 5% goat serum, 5% DMSO in PBST and incubated for 24h at 4°C in movement. Embryos were washed in PBST, cleared for five days in a solution of Urea 4M with 20% glycerol (Hama et al. 2011) and then photographed in a fluorescent stereoscopic microscope (Nikon). Negative controls for the secondary antibodies were performed on embryos processed without pre-incubation with primary antibodies and did not exhibited detectable fluorescence.

#### (e) Embryo Paralysis

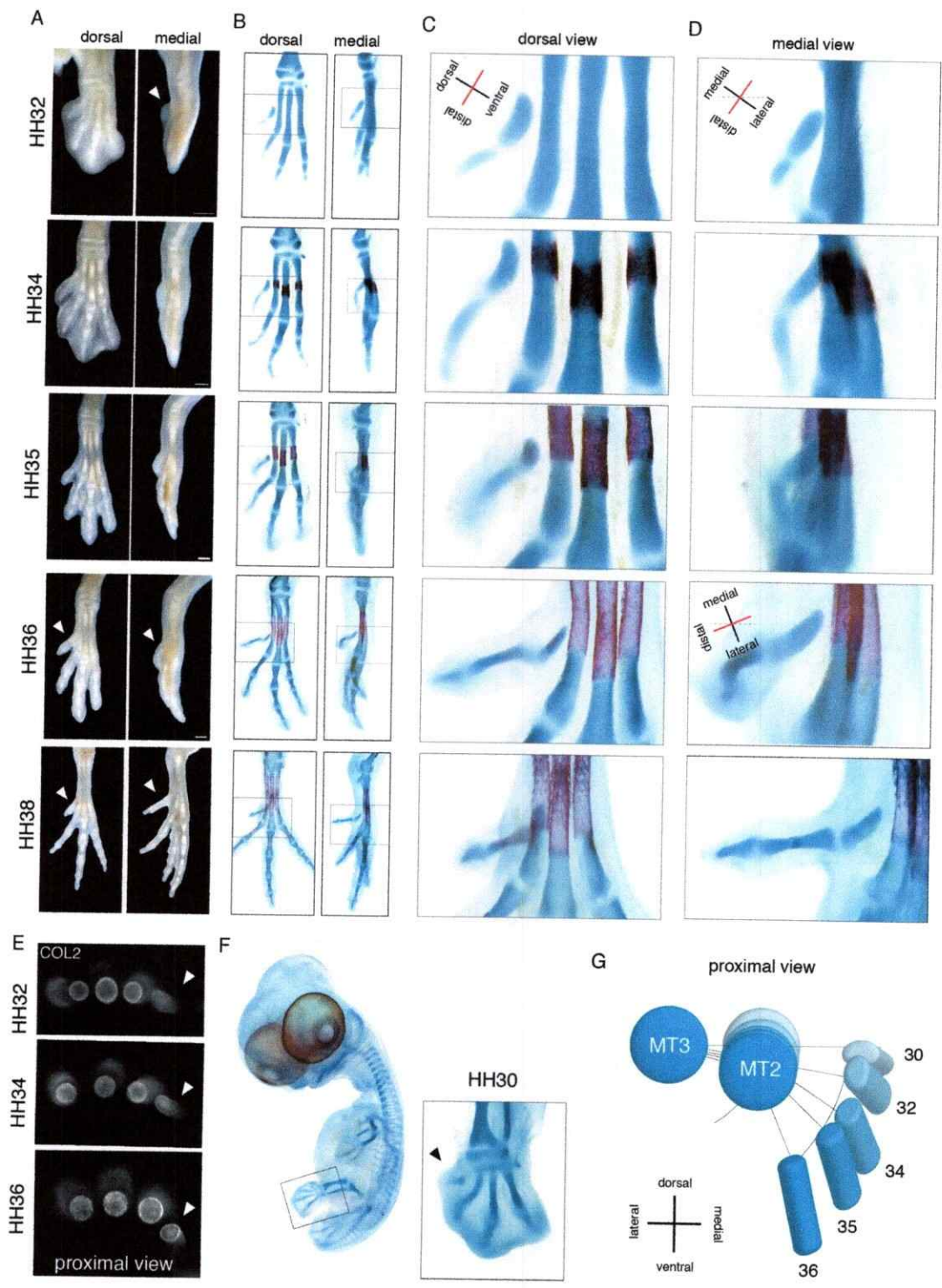
We treated seventy-two chicken embryos at HH34 in three

independent rounds of experiments. Embryos were paralyzed by the application of 100µl of a solution of 1,5g/ml of Decamethonium bromide diluted in PBS (rigid paralysis) (Hall 1975, Pitsillides 2006) or 200µl of a solution of 5mg/ml Pancuronium bromide diluted in PBS, followed by a second application of 100µl two days later (flaccid paralysis) (Nowlan et al. 2010). Control embryos received the same amount of the vehicle, PBS. The drugs were delivered with a micropipette through a small hole in the shell above the air camera. Eggs were sealed with adhesive tape and re-incubated until the appropriate stages, verified by morphological criteria.

## 2.3. Results

### (a) Shifts in position of the hallux during development

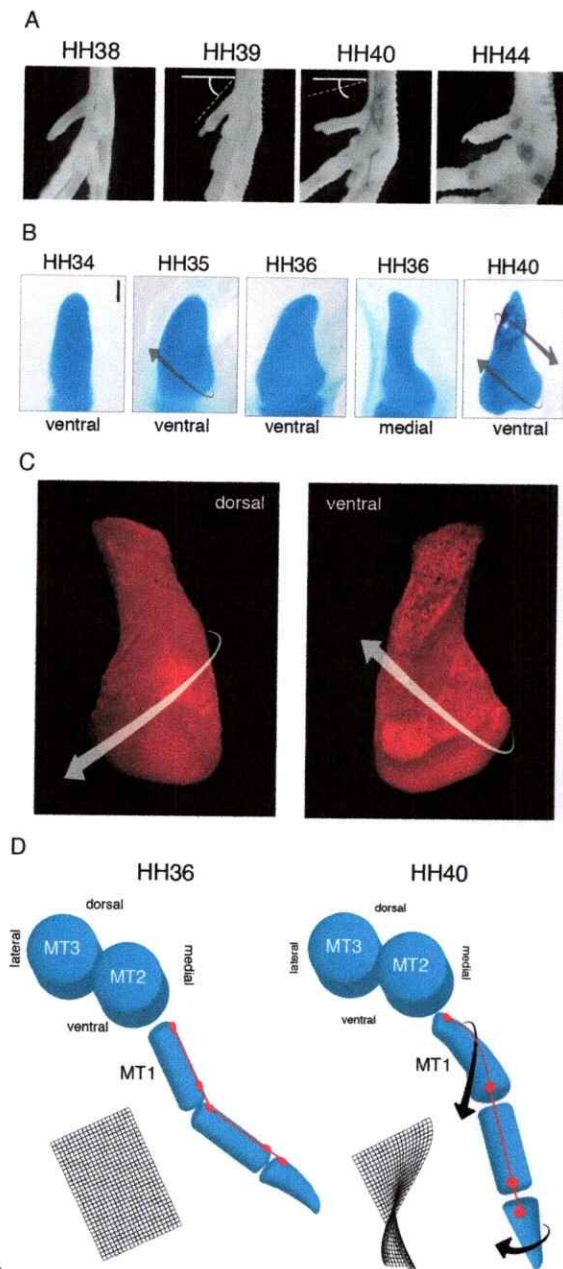
The cartilaginous precursor of Mt1 originates in the medial side of the foot, almost perpendicular to the limb main axis (HH30, figure 3F, black arrowhead). A change in orientation of Mt1 begins at HH32, as the entire hallux becomes tilted, with its distal end slightly displaced towards ventral (backwards, figure 3A, white arrowhead), and rotates such that its ventral side comes to face the lateral side of the foot, much like a human thumb (see HH32, figures 3B and 3D). At HH34 and HH35, hallux phalanges form an angle with mt1, pointing towards distal while mt1 remains slightly tilted, giving the hallux a distinctive "L" shaped appearance in medial view (figures 3C and 3D). At stage HH36, the phalanges of D1 are re-oriented again, such that the entire hallux becomes perpendicular to the main limb axis, its lateral side facing distal, in an elevated, spur-like orientation (figure 3D). Also at HH36, the proximal contact of Mt1 to Mt2 moves markedly towards the ventral face of Mt2 (figures 3D and 3E). At HH38 an opposable hallux has been attained: It is no longer spur-like, presenting a backward orientation (i.e., towards ventral Figure 3C and 3D) and its lateral side faces the medial side of the foot (Figure 1F). Figure 3G summarizes the changes in the orientation of Mt1 with respect to Mt2 in successive stages of development.



**Figure 2.3. The displacement of the hallux.** (A) Dorsal and medial views of the feet of *Coturnix japonica* showing the external morphology during the development of the hallux. Scale bar: 1mm; (B) Dorsal and medial views of Alcian Blue/Alizarin Red stained feet skeletons of *Coturnix japonica* showing changes in the orientation of the hallux (black arrowheads); (C) Detail of hallux skeleton in dorsal view; (D) Detail of hallux skeleton in medial view; (E) Cranial view of *Coturnix japonica* feet sectioned near the level of MT1 articulation and immunostained against COLII showing changes in the orientation of the Mt1 (white arrowheads); (F) Quail embryo stained with Alcian Blue, showing the small size and position of the hallux in the early stages of cartilage development (HH30); (G) Diagram picturing the changing orientation of MT1 between HH32 and HH36 in relation to MT3 and MT2.

#### (b) The torsion of Mt1

In quail and chicken embryos at HH35, the long axis of Mt1 is straight. At HH36 it becomes slightly twisted at its proximal end, and at HH40 torsion is conspicuous along its entire axis (figure 4B), similar to the completely ossified Mt1 of a juvenile chicken (figure 4C). As described above, at HH36 the lateral side of the Mt1 is facing distal (figures 3D and 4D, red line). The torsion of Mt1 is responsible for the change from this orientation to a completely opposable digit: as Mt1 twists, the lateral side of D1 changes from facing distal to facing medial (figure 4D, red line). The torsion is also responsible for the backward projection of the phalanges from their previously medial, spur-like orientation (figure 4A)



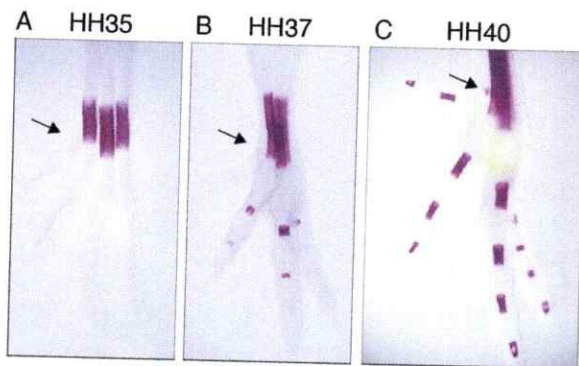
**Figure 2.4. The torsion of MT1** (A) Lateral views of left feet of *Coturnix japonica* late embryos showing the rotation and elevation the hallux (white lines); (B) Ventral views of Alcian Blue/Alizarin Red stained Mt1 of chicken embryos showing the onset of its torsion. Arrow indicate the direction of torsion;(C) Alizarin stained Mt1 from a juvenile *Gallus gallus* in dorsal and ventral view. Arrows indicate the direction of torsion; (D) Diagram picturing from proximal view the torsion orientation of Mt1.

(c) **Maturation and ossification of Mt1 is delayed and begins at its proximal end.**

The ossification of metatarsals 2, 3, and 4 begins around HH34 and their diaphyses are well ossified at HH37, covering over 50% of each of the three metatarsals (figure 5A and 5B). Proximal phalanges begin to ossify at this stage and at HH40 almost all are ossified (figure 5C). In contrast, ossification of Mt1 begins only at HH40, being the last element of the leg – except for the tarsals – to ossify. Moreover, ossification starts at an eccentric position, in its slender proximal tip (Figure 5C, black arrow).

In order to understand the absence of the proximal epiphysis and its possible influence in the development of an opposable hallux, we investigated the early differentiation of Mt1 cartilage using whole mount immunofluorescence assays for markers of skeleton development. Metatarsals are long bones that differentiate in a process called endochondral ossification – the inner replacement of a cartilaginous scaffold by bone. Cartilage is initially composed by immature chondrocytes, characterized by the production of collagen type IIA (COLII) (St-Jacques et al. 1999). Cartilage maturation begins when cells in the center of the element become flattened, and start to produce Indian hedgehog protein (IHH) (Vortkamp et al. 1996, Kobayashi et al. 2002). Those cells later exit the cell cycle, begin to grow (hypertrophy), stop

producing COLII and produce collagen type X (COLX) (Minina et al. 2001). Eventually, the hypertrophic cells die by apoptosis, leaving their extracellular matrix for ossification and remodelling. This dynamic of expansion from the center towards the ends generates two fronts of differentiation – the growth plates (Kronenberg 2003).



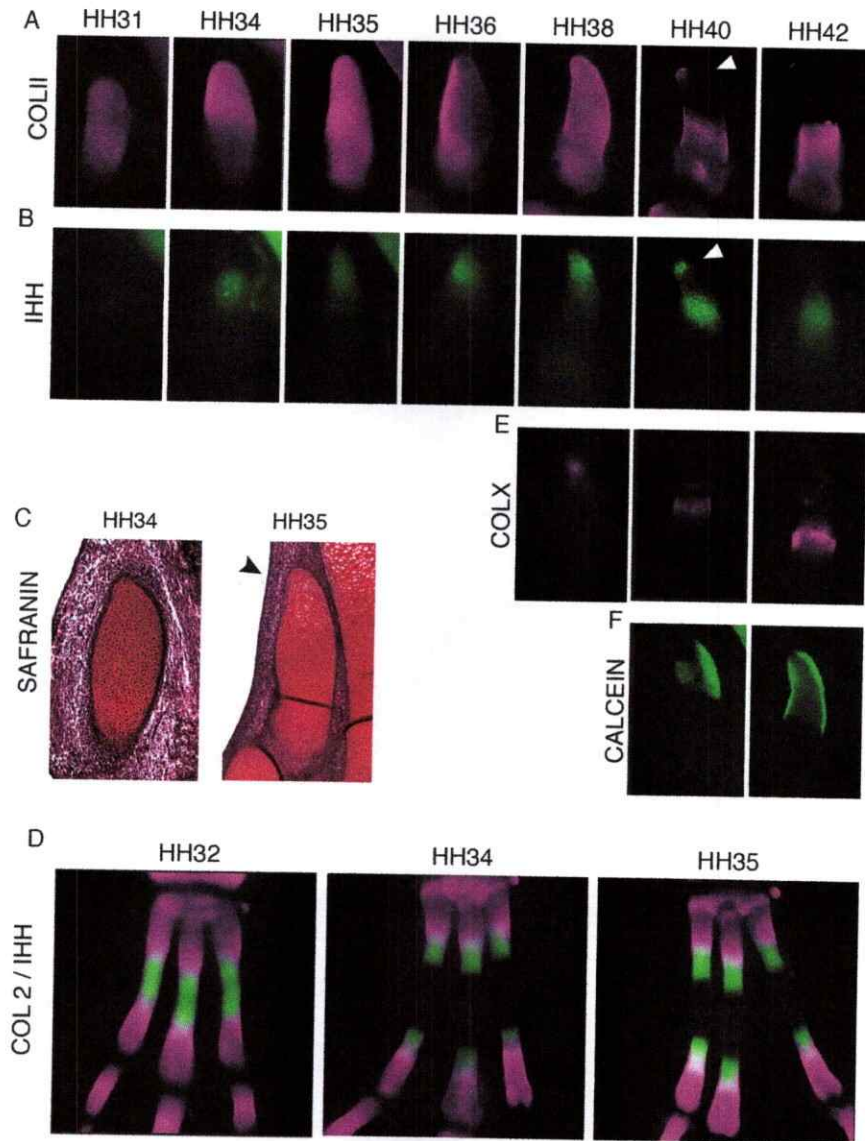
**Figure 2.5: MT1 ossifies late in relation to other foot bones.** Red alizarin staining of quail embryonic foot shows that the ossification of Mt1 (black arrow) is delayed in relation to other metatarsals; (A) HH35, (B) HH37, and (C) HH39.

Compared to the other three avian metatarsals, Mt1 is a small element since the beginning of chondrogenesis (figures 3F and 6A). The size difference increases at HH32, when the other metatarsi elongate conspicuously and Mt1 keeps its small elliptic shape. The presence of COLII as a marker of immature chondrocytes, is detected in the entire element as late as HH38, indicating its slow differentiation (figure 6A). On the other hand, metatarsals 2, 3, and 4 exhibit large areas at their centers lacking COLII (an indication of cartilage maturation) since HH34 (figure 6D). A similar COLII-free area appears in Mt1 only at HH40, when COLII becomes restricted to the distal half



of the element (figure 6A), except for a very small domain of COLII preserved in the proximal end at HH40 (figure 6A, white arrowhead), that has almost disappeared at HH42. IHH production starts in the proximal end of Mt1 at HH34 (figure 6B). The cartilage quickly changes its form, becoming conical at the proximal end at HH35. Histological sections show that hypertrophic chondrocytes appear at the distal extreme at this stage (Figure 6C). A single domain of IHH is maintained in the proximal end until HH38 and, at HH40, two domains of IHH are discernable – a large one in the center and a small one in the proximal extreme (figure 6B, white arrowhead). However, this proximal spot of IHH production soon disappears, and a single domain of IHH is maintained in the center of Mt1 at HH42 (figure 6B), whereas metatarsals 2, 3 and 4 sustain two domains of IHH since HH34 (Figure 6D). COLX synthesis begins in the proximal end of Mt1 at HH38 and advances towards distal in the next stages (figure 6E). Calcification starts in the proximal end at HH40, except by a small area corresponding to the small proximal domains of COLII and IHH at HH38. The distal half of Mt1 is covered by collar bone at HH42 (figure 6F).

The expression domains of these three markers (COLII, IHH, COLX) and Calcein staining show that Mt1 differentiation starts late and in an



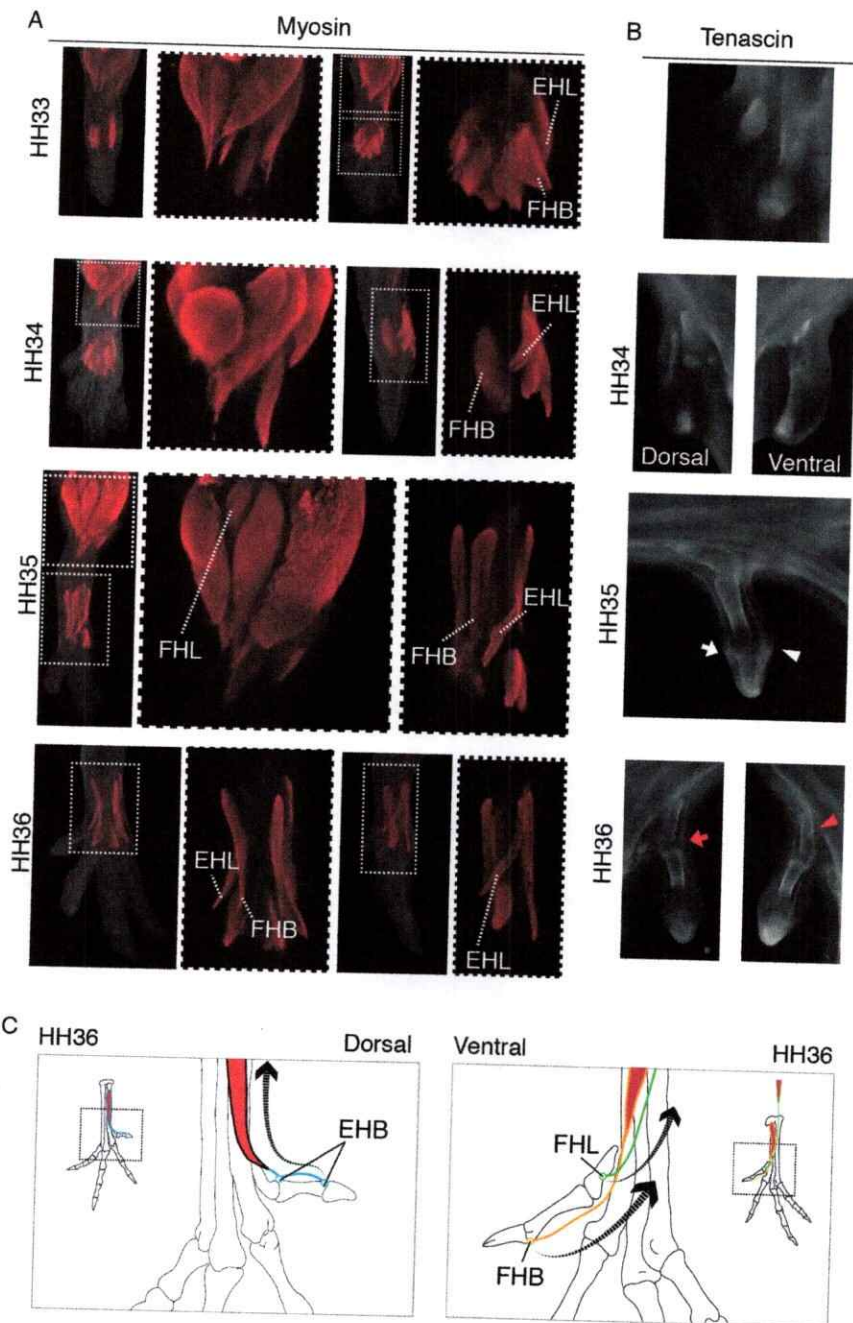
**Figure 2.6. The endochondral ossification of Mt1:** (A) COLII expression during the development of Mt1 indicates the distribution of immature chondrocytes. White arrowhead indicates the vestigial epiphysis; (B) IHH expression during the development of Mt1 indicates the distribution of pre-hypertrophic chondrocytes. White arrowhead indicates the vestigial epiphysis; (C) Paraffin sections of Mt1 at HH34 and HH35 show the appearance of hypertrophic cells in the proximal side at HH35 (black arrowhead); (D) Dorsal view of COLII (purple) and IHH (green) expression during the development of quail left metatarsi. (E) COLX expression during the development of Mt1 indicates the distribution of hypertrophic chondrocytes; (F) Calcein staining during the development of Mt1 showing the beginning of ossification.

eccentric position, near the proximal end. A vestigial proximal epiphysis is generated but it soon disappears. This process results in a bone with a small diaphysis and just one growth zone in the distal end. As a consequence of this arrested differentiation, the Mt1 is completely cartilaginous until an exceptionally late stage for a long bone (HH38) and, even after the beginning of ossification, the cartilaginous epiphysis occupies around half its total length (HH42).

#### (d) Muscle development and activity in the hallux

Mt1 forms as a straight cartilage until HH35. Torsion begins at HH36 and it is markedly twisted at HH38. It is an immature cartilage throughout this process (Figure 4B). We investigate if mechanical forces generated by the muscular system when it is still in an early phase of cartilage differentiation could be responsible for this torsion.

Three muscles – two flexors and one extensor – control the avian hallux. The muscle *flexor hallucis longus* (FHL) is the only extrinsic muscle of the hallux – its belly is situated outside the foot, in the ventral shank; its tendon goes through the lateral hypotarsus and crosses obliquely to the ventral tarsometatarsus, and its principal insertion is in the ventral base of the unguis phalanx (figure 6C). Quail embryos exhibit a well-individualized FHL at HH33,



**Figure 2.7. The development of hind limb muscles and tendons** (A) Whole-mount immunofluorescence against myosin in quail embryos between HH33 and HH36 reveals the development of *musculus extensor hallucis longus* (EHL), *m. flexor hallucis longus* (FHL) and *m. flexor hallucis brevis* (FHB); (B) Whole-mount immunofluorescence against tenascin in quail embryos reveals the insertion of EHL (arrow), FHL (arrow), and FHB (arrow); (C) Schematic representation of the foot musculoskeletal system at HH36 and the muscular forces proposed to provoke the orientation change of Mt1.

as can be visualized by whole-mount immunofluorescence against Myosin (figure 6A). Its insertion is discernible by the expression of Tenascin at HH35 (figure 6B, white arrowhead).

The other flexor of the hallux, the muscle *flexor hallucis brevis* (FHB), is located in the medio-ventral side of the foot; its tendon inserts in the ventral side of Mt1 or at the base of the proximal phalange, depending on the species (George and Berger 1966). The muscle originates from a common mass of muscular fibre for all ventral intrinsic muscles (Kardon 1998). Its distal part is discernible since HH33 (figure 6A); its insertion in the ventral Mt1 is visible at HH35 (Figure 6B, red arrowhead).

The *musculus extensor hallucis longus* (EHL) is the only extensor of the hallux. It arises from the dorso-medial border of the Mt2 and its main insertion is in the base of the unguis phalange. A secondary insertion in the base of the proximal phalange is common. In quail embryos, it separates from the dorsal mass of intrinsic muscles at HH35, when its insertion is already visible (figure 6C, white arrow); a secondary insertion is present at HH36 (figure 6B, red arrow).

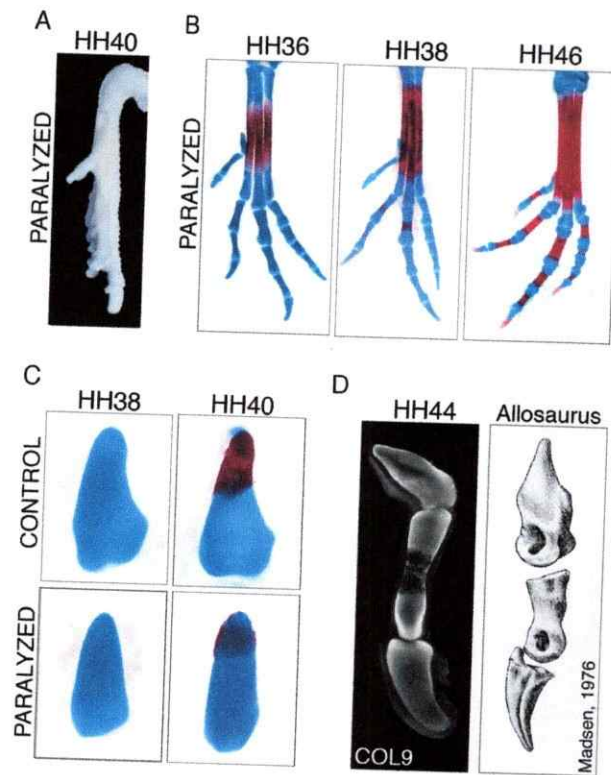
Our data show that the musculoskeletal system is completely connected to the hallux at HH36, when torsion begins. The onset of digit movements occurs as soon as the muscular system is anatomically functional

(Gottlieb and Kuo 1965). Digits are immobile until HH34, when the movements of the foot are restricted to the ankle joint. The first digit movements – synchronous extensions of all digits – appear at HH35, probably due to the action of the *musculus extensor digiti longus*. The flexion of the digits, including the hallux, starts at HH36 (video suppl. 1).

(e) The absence of muscular action on the hallux

The temporal dynamics of muscle development and activity upon the cartilaginous hallux suggest that these processes are involved in the torsion of Mt1. We tested this hypothesis by paralyzing chick embryos before the twisting of Mt1 (HH34). Eggs were injected with Decamethonium bromide to produce rigid paralysis and Pancuronium bromide to produce flaccid paralysis. Paralyzed embryos – both flaccid and rigid – show normal ventral displacement of Mt1 (Figure 8B). Therefore, the change of the articulation from the medial to the ventral side of Mt2 is not influenced by muscular activity. Nevertheless, in paralyzed embryos the ventral side of the hallux faces the medial side of the foot and its long axis lays parallel to the long axis of Mt2 at HH40, similar to control embryos before the torsion of the hallux (figure 8A and 8B). Consistent with this, Mt1 of paralyzed embryos fails to twist and has a straight shape (figure 8C). The morphology of Mt1 in paralyzed chick embryos is remarkably similar to that of early tetanuran dinosaurs like *Allosaurus*

(Madsen 1976) (figure 8D), having a straight shaft and a ventral side facing the medial side of Mt2.



**Figure 2.8. Paralyzed embryos fail to develop an opposable hallux**

(A) Paralyzed embryos of *Gallus gallus* at HH40 showing the maintenance of the medial orientation of the hallux; (B) Paralyzed embryos of *Gallus gallus* exhibit the hallux articulated to the ventral Mt2 but not opposable; (C) Mt1 of paralyzed embryos is straight, as opposed to control embryos; (D) Mt1 of paralyzed embryos of *Gallus gallus* at HH44 immunostained for Col9 resembles the Mt1 of early theropod dinosaurs, as *Allosaurus*



## 2.4. Discussion

The ontogenetic acquisition of an opposable hallux in birds goes through a sequence of transitory orientations. At early stages, Mt1 rotates and its ventral side points towards lateral, like a human thumb (Figure 9A). It is similar to the orientation known for most non-avian tetanuran fossils (Norell and Makovicky 1997, Carrano et al. 2012). Subsequently, the contact between Mt1 and Mt2 moves from the medial to the ventral side of Mt2, but the orientation of the hallux does not change: its ventral side continues pointing towards the lateral side of the limb, with Mt1 abducted almost perpendicular to the tarsometatarsus (Figure 9B-D). This perpendicular orientation is common in embryos of other species like ducks (*Anas platyrhynchos*), zebra finches (*Taeniopygia guttata*), and budgerigars (*Melopsittacus undulatus*) (data not shown), and some extant birds, like gulls, conserve this orientation in adulthood (Figure 1H). The torsion of the Mt1 begins with the hallux in the perpendicular orientation and goes from medial-to-lateral. Its main effect is to change the hallux's lateral side from facing distally to medially, fully reversing digit orientation (figure 4D and 9E).

The ontogenetic steps in the reorientation of the hallux suggest that the torsion of Mt1 – not the displacement of the articulation – is the main



cause of the development of the opposable hallux in extant birds. Indeed, birds that have the hallux articulated to the medial face of Mt2 (e.g. Sulidae, figure 2J) can exhibit an Mt1 as twisted as birds that have the hallux articulated to the ventral Mt2 (e.g. Passeriformes, figure 2G and 2L). Moreover, the ventral position of the Mt1 articulation is known to be present in only some extant birds, and could have evolved much later than the opposability of the hallux (see below). Therefore, we identify the acquisition of a twisted Mt1 as the central characteristic that allowed the evolution of an opposable hallux.

Avian Mt1 has only one epiphysis, and, although most long bones have two, bones that have a single epiphysis are not unusual. Mammalian metatarsals, for example, lack one epiphysis (the distal of the thumb and the proximal of other toes) and the avian fibula lacks the distal epiphysis. Nevertheless, in both cases, the beginning of ossification is not eccentric and the epiphysis is lost as the growth zone advances (Müller 1989, Reno et al. 2006). Avian Mt1 differs from other bones that have lost an epiphysis in two main aspects: (1) chondrocyte differentiation begins late and asymmetrically, at the proximal end, and (2) chondrocyte differentiation leads to an ossified shaft that is smaller than the cartilaginous epiphysis. Thus, differentiation of Mt1 is significantly slowed down, remaining cartilaginous until much later stages than other metatarsi. This could allow torsion of its long axis, which otherwise may have been impossible if mechanical forces were exerted at a later stage of

cartilage differentiation, or over an already ossified bone.

The musculoskeletal system controlling the hallux is developed and active as from HH36 – the period when Mt1 torsion begins and it is still an immature cartilage. As the muscular activity continues, the hallux is re-oriented and Mt1 becomes twisted before it ossifies. The temporal coherence of the two events supports our interpretation that muscular activity is responsible for the torsion of the Mt1. The pharmacological paralysis of chicken embryos before Mt1 torsion avoids its torsion and the reorientation of the hallux. This experiment confirms that embryonic muscular activity is necessary for the development of a fully opposable hallux. Further, this experiment phenocopies the straight, non-twisted morphology of Mt1 observed in ancestors of birds such as early tetanuran dinosaurs. The ancestral topology of musculature controlling the hallux in these ancestors is inferred to be similar to the topology found in modern birds (Dilkes 1999, Hutchinson 2002). Therefore, the development of a twisted Mt1 probably was allowed by the delay of Mt1 differentiation, and not by the evolution of new muscles or insertions. Nevertheless, we cannot rule out the possibility that embryonic motility begins earlier or is more intense in modern birds than in their ancestors, and that this anticipated or amplified the influence of muscles on Mt1 development. Modern petrels and penguins (Hudson 1937, George and Berger 1966) have secondarily re-evolved a straight Mt1 (Figure 1B and E).

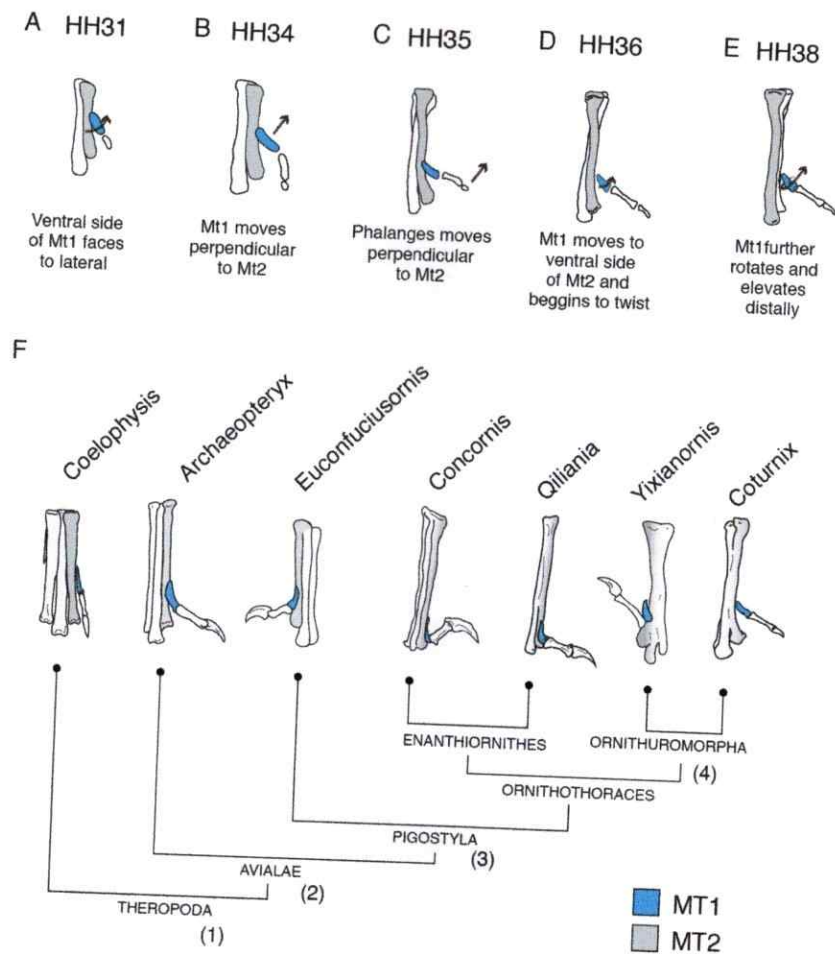
Importantly, in these taxa, the FHL and EHL muscles are absent (Raikow 1985), which again is consistent with the role of muscular activity in the development of the shape of Mt1.

Taken together, we propose that the twisting of Mt1 is crucial for the development of a fully opposable hallux, and is caused by the action of muscles upon a cartilaginous precursor whose differentiation and ossification is remarkably delayed. This developmental model is insightful about the morphology of fossil taxa related to modern birds. Cladistic analyses of Mesozoic birds often code three ordered states for the Mt1 (Clarke et al. 2006, O'Connor et al. 2009, Godefroit et al. 2013): "*Metatarsal I: (0) straight; (1), ventral surface convex 'J shaped'; (2) deflected and twisted such that the ventromedial surface is concave proximal to trochlear surface for phalanx I.*" The state (0) is exhibited by *Archaeopteryx* and all other non-avian Paraves representatives, whose articulated Mt1 are preserved ((e.g. Norell and Makovicky 1997, Xu et al. 2000, Zhang et al. 2002, Xu et al. 2011, Agnolín and Novas 2013, Godefroit et al. 2013)). Long-tailed birds from the Early Cretaceous of China as *Jeholornis prima* (Zhou and Zhang 2002, Zhou and Zhang 2007), *Dalianraptor cuhe* (Gao and Liu 2005) and *Shenzhouraptor sinensis* (Ji et al. 2002) also exhibit this plesiomorphic state. The state (1) is exhibited by basal Pygostilia, such as Confuciusornithidae (Chiappe et al. 1999, Hou et al. 1999, Zhang et al. 2009) and Enantiornithes (Chiappe et al. 2002, Sereno et al. 2002). They represent

the first evidence for a change in the orientation of the hallux. A similar morphology is found in basal Ornithuromorpha (Clarke et al. 2006) (O'Connor et al. 2011). The state (3) is known only in Ornithurae, including extant birds (Figure 9E).

Birds are paedomorphic when compared to non-avian dinosaurs (Bhullar et al. 2012), and the evolution of their opposable hallux occurred concomitant to other major developmental transformations in the skeleton, such as the acquisition of a pygostyle and the fusion of tarsals and metatarsals (O'Connor et al. 2011). The ontogeny of the avian opposable hallux suggests that its evolution was intimately related to the acquisition of a malleable Mt1. We propose that the acquisition of a more malleable Mt1 was allowed by its delayed differentiation associated to other heterochronic modifications of the skeleton exhibited by pygostylian birds (Figure 9F). Non-avian theropods and tailed birds conserve a straight Mt1, similar to the one observed in HH32 and in late stages of paralyzed embryos. Since the avian muscular system controlling the digit is inferred to be functionally similar to non-avian theropods, this suggests that muscular activity was unable to produce torsion, perhaps because Mt1 was more differentiated and less plastic upon the onset of embryonic motility. In early Pygostylia, the J-shaped Mt1 suggests that Mt1 differentiation was delayed to a somewhat lesser extent than modern birds, such that muscle activity encountered an already differentiated proximal end,

but a still immature, plastic distal epiphysis, resulting in a J-shaped bone. Finally, the crosswise torsion observed in modern birds would have been possible after a further delay in differentiation, which allowed torsion of the entire Mt1 (Figure 9E).



**Figure 9: A developmental model for the evolution of the avian opposable hallux.** (A) HH31: The hallux flexes and its ventral side faces Mt2; (B) HH34: Mt1 is elevated, perpendicular to Mt2; (C) HH35: D1 phalanges are also elevated and the entire hallux becomes perpendicular to Mt2; (D) HH36: Mt1 moves from the medial to the ventral side of Mt2 and begins to twist; (E) HH38: Mt1 becomes conspicuously twisted and the ventral side of the hallux faces distal, as opposed to the other three digits. (F) The evolutionary history of the hallux in the lineage leading to modern birds. 1: Neotheropoda lost Mt1 proximal epiphysis; 2: Paraves displaced Mt1 articulation to the distal Mt2; 3: Pygostyla delayed the ossification of Mt1, allowing its distal bending by embryonic muscular activity; 4: Ornithuromorpha further delayed Mt1 ossification, allowing its torsion by embryonic muscular activity.

## 2.5 Conclusion

We have shown that the development of the avian opposable hallux was allowed by the arresting of Mt1 differentiation and the action of muscles over the skeleton during the embryonic period. The importance of forces exerted by embryonic muscular systems in the development of specific skeletal traits has been demonstrated in different models (Newman and Muller 2005, Nowlan et al. 2008, Nowlan et al. 2010, Schweitzer et al. 2010). In birds, for example, the cnemial crest and the sternal quill are absent in paralyzed chicken embryos (Müller and Streicher 1989, Hall and Herring 1990). Importantly, our developmental data shows a compelling correspondence with documented evolutionary changes, both at the origin of the hallux and in its subsequent evolution among modern birds. This emphasizes how the investigation of variations in the rates of ontogenetic processes and interactions at different levels of biological organization are required for a proper understanding of evolution, beyond other typically pursued research goals such as the identification of mutations and ecological adaptations.

### The Developmental Origin of Zygodactyl Feet and its Possible Loss in the Evolution of Passeriformes

**Abstract:** The zygodactyl orientation of toes (digits II and III pointing forward, digits I and IV pointing backwards) evolved independently in different extant bird taxa. To understand the origin of this trait in modern birds we investigated the development of the zygodactyl foot of the budgerigar (Psittaciformes). We compared its muscular development with that of the anisodactyl quail (Galliformes) and show that while the *musculus abductor digiti IV* (ABDIV) becomes strongly developed at HH36 in both species, the *musculus extensor brevis digiti IV* (EBDIV) degenerates and almost disappears only in the budgerigar. The asymmetric action of those muscles early in the development of the budgerigar foot causes retroversion of digit IV. Both molecular phylogenetic analysis and paleontological information suggest that the ancestor of passerines could have been zygodactyl. We followed the development of the zebra finch (Passeriformes) foot muscles and found that in this species both the primordia of the ABDIV and of the EBDIV fail to develop. These data suggest that loss of asymmetric forces of muscular activity exerted on digit IV, caused by the absence of the ABDIV, could have resulted in secondary anisodactyly in Passeriformes.

**Keywords:** anisodactyly; myogenesis; Passeriformes; Psittaciformes; zygodactily.



### 3.1 Introduction

Differences in the morphology of the foot are among the main factors that allowed the specialization of the avian leg (for a review see (Raikow 1985)). The ancestral condition to extant birds was a four toed cursorial foot (but functionally tridactyl) as observed in non-avian theropods (Ostrom 1976, Padian and Chiappe 1998). The anisodactyl foot – where the digits II, III, and IV are oriented forward while digit I is oriented backward – evolved in early Avialae by the retroversion of the hallux (Middleton 2001, Mayr et al. 2005). In extant birds, modifications of the anisodactyl foot produced similar morphologies independently in different taxa. Several families reduced or lost the hallux. Palmated feet with webs between the digits evolved in many species of aquatic birds. Further anatomical variation was generated by changes in the orientation of the digits. Zygodactyl foot have digits I and IV oriented backward and digits II and III oriented forward, and have evolved independently by the backward orientation of digit IV (dIV) in at least three extant clades: Cuculidae (cuckoos), Psittaciformes (parrots) and Piciformes (woodpeckers and allies). Some birds are semi-zygodactyl and can facultatively change the orientation of dIV, like Musophagidae (turacos), Pandionidae (ospreys), Strigiformes (owls) and Coliidae (mousebirds). An arrangement that is functionally similar to zygodactyly, named heterodactyly, has evolved in

Trogoniformes (trogons), where digits I and II are oriented backward and digits III and IV are oriented forward. Finally, swifts (Apodidae) have a pamprodactyl foot, a condition where all four digits can be oriented forward.

It is remarkable that almost every variation of the anisodactyl foot has evolved independently in different lineages. This suggests that not only ecological demands, but also developmental factors could have driven the direction of evolutionary changes (Arthur 2004, Newman and Muller 2005). To understand the nature of those factors, we explored one of these transformations – the origin of the zygodactyl feet – from a developmental perspective.

Previous studies about the origin of zygodactyly have focused on its functionality and on its underlying musculoskeletal organization. Zygodactyly has often been considered as an adaptation for climbing, perching or manipulation (Bock and Miller 1959, Raikow 1985, Zelenkov 2007, Mayr 2009). However, taking into account the diversity of niches occupied by extant zygodactyl species and also the fact that those same niches are occupied by non-zygodactyl birds, it is not straightforward to find a common evolutionary scenario to explain its origin. Moreover, anatomical comparison of the musculoskeletal system has shown that there is not a common anatomy to all zygodactyl feet (Steinbacher 1935, Berger 1952, Swierczewski and Raikow 1981, Berman 1984), making it difficult to recognize which elements are

muscles were macerated with 2% KOH. The embryos were cleared in a series of Glycerol.

### (c) Immunohistochemistry and Immunofluorescence

Four embryos for each stage were used for immunofluorescence against myosin (*C. japonica*, *M. undulatus*, and *T. guttata*) and tenascin (*M. undulatus*). Embryos were fixed in Dent's fix (4:1 methanol:DMSO) for 2 hours, dehydrated in a series of methanol, and left for at least 12 hours at -80°C. Then, the specimens were bleached in Dent's bleaching (4:1:1 methanol:DMSO:H<sub>2</sub>O<sub>2</sub>) for 24 hours at room temperature. They were rehydrated in phosphate buffer with 1% triton X-100 (Sigma) (PBST). Primary antibodies against myosin (MF-20 from DSHB, Iowa) and tenascin (M1-B4 from DSHB, Iowa) were diluted 1:20 in PBST; 5% horse serum and 5% DMSO. Embryos were kept in primary antibody for 48 hour at 4°C in an orbital shaker. Embryos were washed in PBST six times for one hour. Secondary antibodies anti-mouse made in donkey coupled to horseradish peroxidase (715-035-150, Jackson ImmunoResearch) or Alexa-Fluor 594 (715-585-150, Jackson ImmunoResearch) were diluted 1:300 in PBST, 2% horse serum and 5% DMSO. Embryos were kept in secondary antibody for 24 hour at 4°C in an orbital shaker. They were washed again in PBST six times for one hour. Embryos labelled with horseradish peroxidase were revealed with

### 3.2. Materials and Methods

#### (a) Animals

Fertilized eggs of Japanese quail (*Coturnix japonica*), zebra finch (*Taenopygia gutatta*), and budgerigar (*Melopsittacus undulatus*) were obtained from colonies at the University of Chile. The eggs were removed from nests and incubated in an automatic incubator at 37.5°C and 60% of humidity. All procedures follow the protocols approved by the ethical committee of the University of Chile. The nomenclature employed for the skeleton, muscles, and tendons follow *Nomina Anatomica Avium* (Baumel et al. 1993). The embryos were staged using the normal table for *Gallus gallus* (Hamburger and Hamilton 1951).

#### (b) Cartilage and Bone Stain

Developmental series for each species investigated were prepared for skeletal staining. Embryos were fixed in 100% methanol for at least two days. Cartilage was stained with a solution of 0.02% Alcian Blue (Sigma-Aldrich) diluted in 5:1 Ethanol/Acetic acid for 24-48 hours. Bone was stained with a solution of 0.02% Alizarin Red (Sigma-Aldrich) diluted in water with 0.5% of KOH for 2 hours. The excess of dye was washed with water and the

specifically related to that arrangement. In this work we investigate the development of the foot musculoskeletal system of a zygodactyl bird – the budgerigar (*Melopsittacus undulatus*) – to discern which factors are causally related to its development and to understand the possible causes of its convergent evolution. For appropriate inference, we also examined the development of the foot of the Japanese quail and zebra finch, two anisodactyl birds with radically different evolutionary histories.

Diaminobenzidine substrate (11718096001, Roche Applied Bioscience). To avoid background, the first 10 minutes of reaction were carried out in ice.

(d) Paralysis

Fourteen budgerigar embryos were pharmacologically paralyzed; eight survived. After candling the egg, a small hole was opened with a needle over the air sac. A single dose of 20 $\mu$ l of a solution containing 2mg/ml of Decamethonium bromide diluted in phosphate (modified from (Hall 1975) was then delivered with a micropipette to embryos at HH31 (Hamburger and Hamilton 1951). The egg was sealed with a glue gun and incubated without movement for four days.

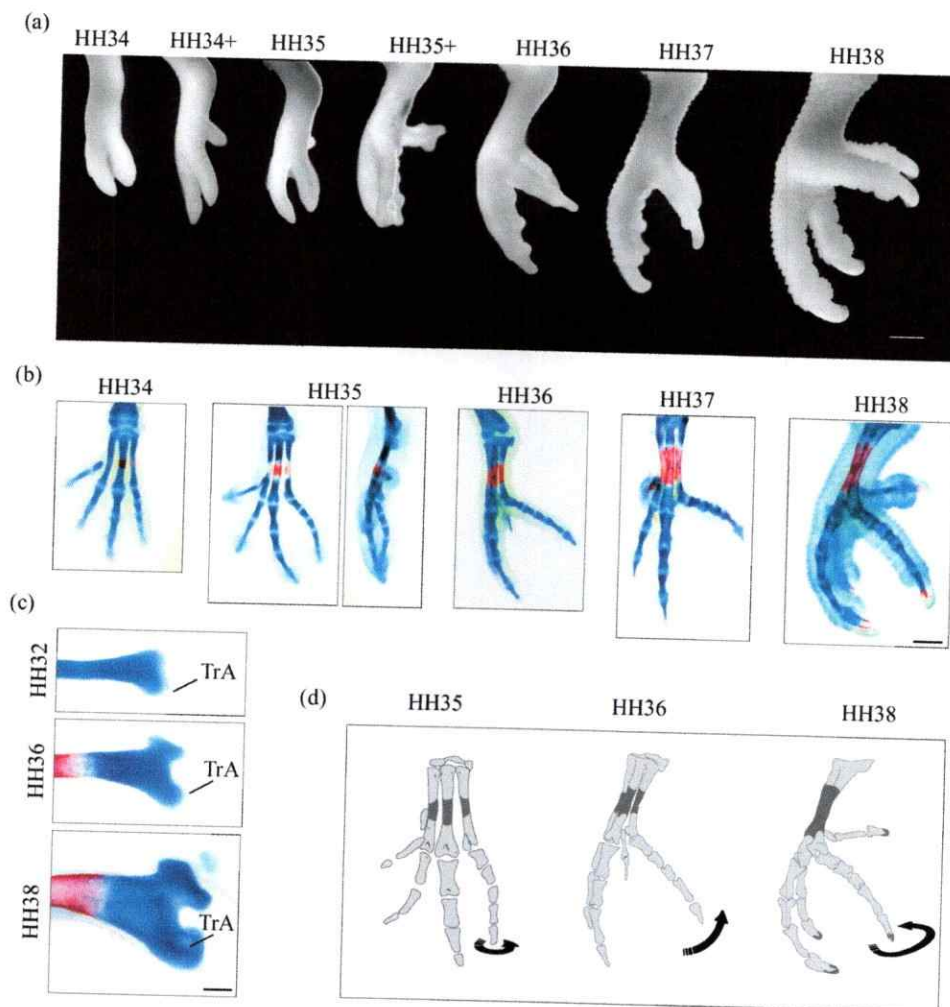
### 3.3. Results

#### (a) Rotation of Digit IV in budgerigar development (Psittaciformes)

The first step to understand the development of the zygodactyl foot was to determine when and how dIV changes its orientation, departing from the development of the anisodactyl foot. Limb development is well known in chicken and quail, and thus these animals yield good anisodactyl models to compare with the budgerigar. In these taxa, the toes develop from cartilages originated in a pad-like flattening of the distal limb bud. Chicken limbs exhibit the cartilages of the four digits at HH31, except for the distal-most phalanges (Hamburger and Hamilton 1951). At this stage, toes are united by mesenchymal tissue and oriented in the same plane. Posteriorly, the tissue between the toes becomes a thin web, the digits elongate conspicuously, and a collar of bone begins to grow in the centre of each metatarsus (HH34). Eventually, the digits separate from each other (HH35), the hallux rotates towards ventral and the primordia of the claws appear (HH36).

We produced a developmental series for budgerigar stained with Alcian Blue and Alizarin Red. At early stages, the development of the foot of budgerigar does not differ from what has been described for the domestic chicken. The first sign of zygodactyly occurs around HH35, when dIV rotates

medio-laterally at the level of the articulation between the metatarsus and the proximal phalanx (figure 1ab). Consequently, the medial side of the phalanges of dIV comes to face dorsal and the lateral side to face ventral. The dIV is then flexed



**Figure 4.1:** (a) The anatomy of budgerigar early foot development; (b) The anatomy of budgerigar early hind limb skeletal development. Cartilages in blue (Alcian Blue); bones in red (Alizarin Red) (c) The *trochlea accessoria* (TrA) at different stages. MtIV has been isolated and photographed in lateral view; (d) Diagram picturing the change of dIV orientation during development. Scale bars: (a) and (b): 1mm; (c) 200 $\mu$ m.

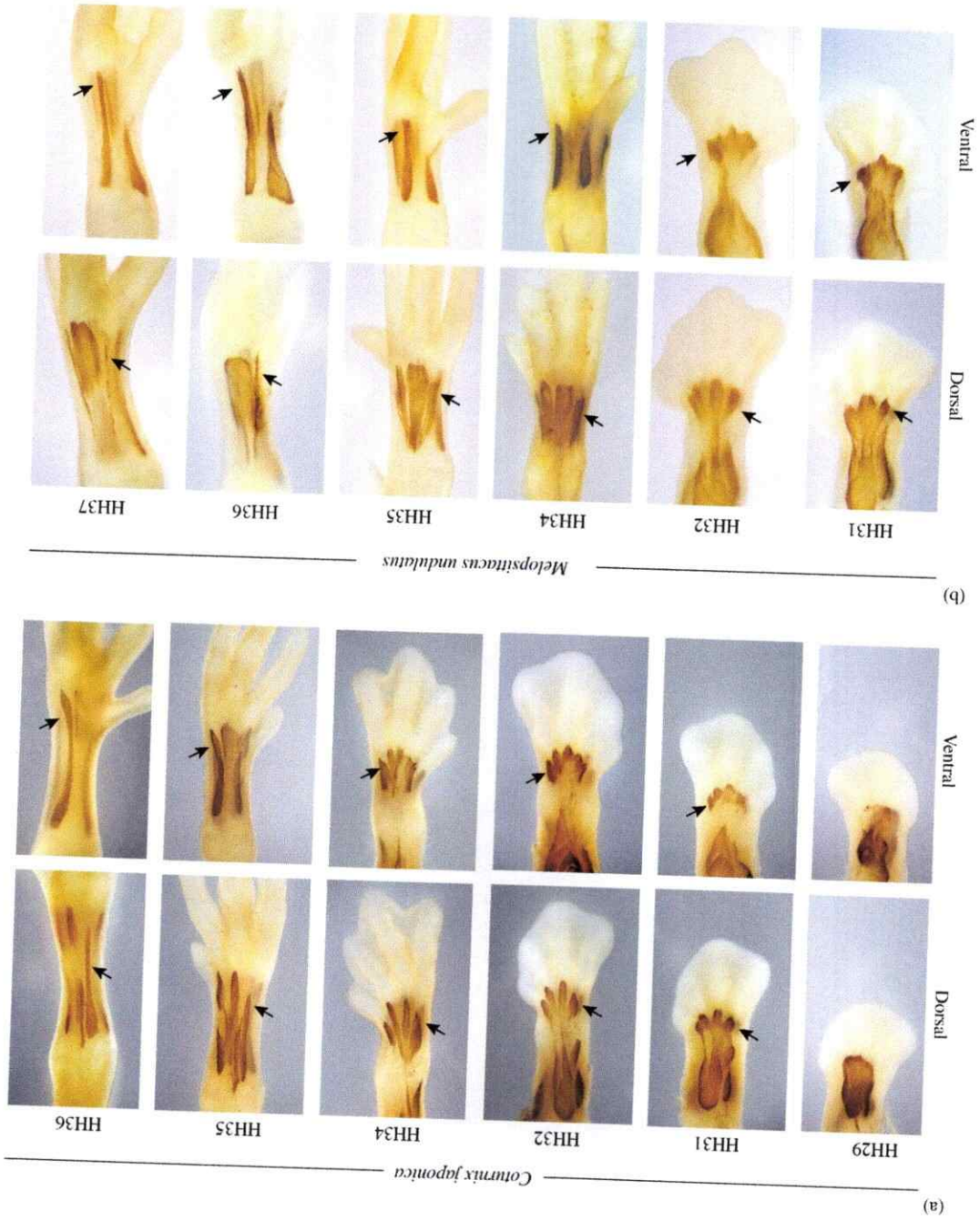


and acquires a right angle in relation to the main axis of the metatarsus (HH36). The morphology at this stage is similar to that of adult semi-zygodactyl birds like owls and touracos. Eventually, dIV rotates further toward medial and the foot becomes fully zygodactyl (HH37). The rotation and flexion of dIV is followed by a ventral outgrowth of the distal end of metatarsal IV. First, it develops a wing-like flange similar to the *trochlea accessoria* (*TrA*) observed in semi-zygodactyl birds (HH36); later it acquires the hooked shape (or *sehnenhalter*) characteristic of the *TrA* of fully zygodactyl birds like Psittaciformes and Piciformes (figure 1c). In summary, during the development of the budgerigar foot, dIV rotates at HH35, flexes at HH36 and further rotates at HH37 (Figure 1d).

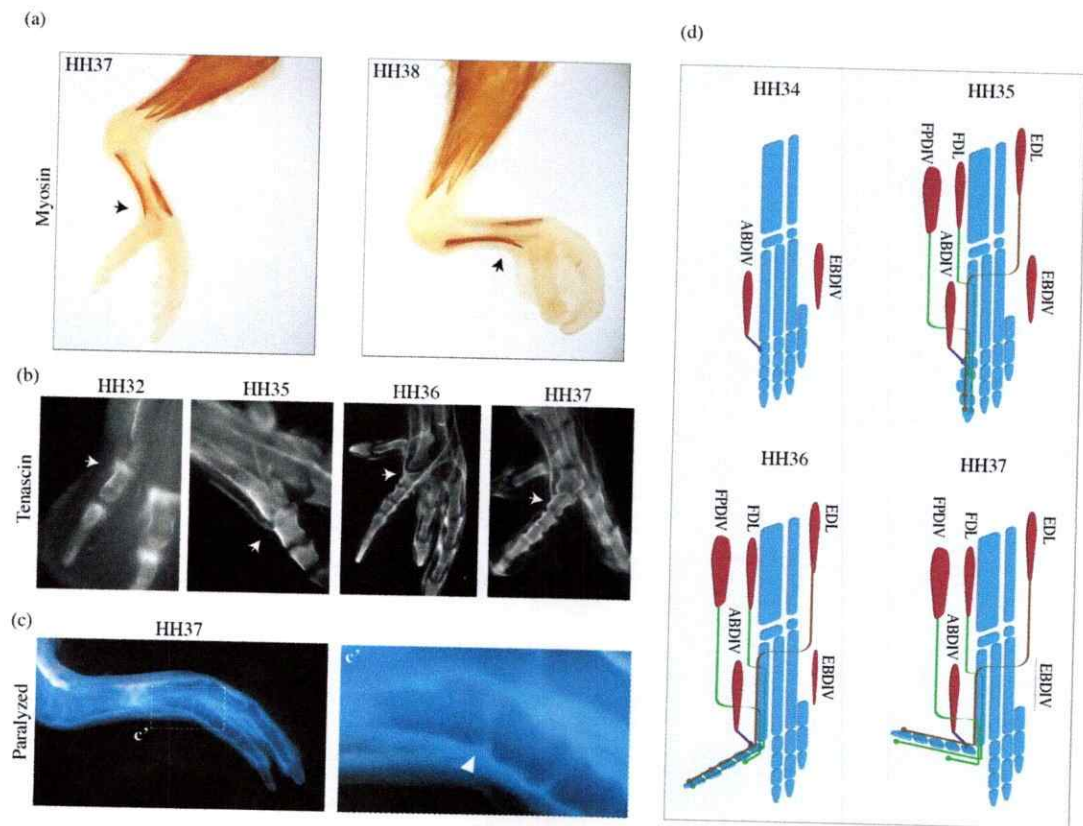
(b) *Extensor brevis digiti IV* is lost in the development of the budgerigar

The time and mode of change in orientation of dIV suggest that early muscular activity could be acting in the transformation of the foot. The early development of avian hind limb muscles has been studied in the domestic chicken (Wortham 1948, Chevallier et al. 1977, Kieny et al. 1988, Kardon 1998). Muscle cell precursors originated in the lateral somite migrate to the limb bud around HH22 (Hayashi and Ozawa 1991). The precursor cells

EBDIV.  
**Figure 4.2.** Immunohistochemical reaction against myosin 2 showing the early development of dorsal and ventral foot muscles in quails (a) and budgerigars (b). Black arrows indicate the



generate a dorsal and a ventral mass of differentiating muscle fibers. Each mass divides, generating successively smaller masses, and eventually each individual muscle around HH35. Movements of the ankle and digits begin at HH32 and at HH35, respectively (Kuo 1932).



**Figure 4.3.** (a) Immunohistochemical reaction against myosin 2 showing the late disposition of the ABDIV (black arrows) in relation to the changes in the orientation of dIV; (b) Immunohistochemical reaction against tenascin showing the insertion of the tendon of the ABDIV (white arrows); (c) Morphology of dIV in paralyzed embryos; (c') detail of the *trochlea accessoria*; (d) Diagram illustrating the development of the musculoskeletal system in the budgerigar. Muscles in red; skeleton in blue. FDL: *flexor digitorum longus*; EDL: *extensor digitorum longus*; FPDIV: *flexor perforatus digiti IV*

The digits are controlled by a complex combination of those individual muscles. Flexor muscles, responsible for grasping movements, are found in the ventral side, while extensor muscles, responsible for releasing, are found in the dorsal side. Adductor and abductor muscles are defined in relation to the limb axis. Therefore, the abductor of dIV is the muscle inserted on the lateral side of dIV, while the abductor of digit II (dII) is inserted on the medial side of dII. The muscles that control the digits can be further classified according to their location into intrinsic and extrinsic muscles. The extrinsic muscles are situated in the crus while the intrinsic muscles are situated in the foot.

The complex combination of muscles and tendons controlling bird toes is highly variable among taxa. There is a large amount of literature concerning this variation, as it has been employed to investigate the high-order phylogeny of birds (Gadow 1882, Hudson 1937, Swierczewski and Raikow 1981). Those studies suggest that for Neornithes the ancestral condition for the muscles controlling dIV is similar to the condition observed in Galliformes, like the chicken and the quail. In those birds, the extension of dIV is controlled by one extrinsic muscle attached to the dorsal side of the distal phalanx (*M. extensor digitorum longus*) and one intrinsic muscle attached to the medial side of the proximal phalanx (*M. extensor brevis digiti IV* -

EBDIV). The medial insertion of EBDIV probably enables it to act also as an adductor. The flexion of dIV is controlled by two extrinsic muscles attached to the distal phalanx (*M. flexor digitorum longus* and *flexor perforatus digiti IV*), while its abduction is produced by a ventral intrinsic muscle inserted in the lateral face of the proximal phalanx (*M. abductor digiti IV* – ABDIV) (figure 3d).

To compare the development of the muscles controlling dIV in the anisodactyl quail and the zygodactyl budgerigar, we examined embryos immunostained for myosin type 2. We found that the development of the extrinsic muscles is very similar in both species. However, the two species differ in the intricate set of fusions, changes in size and displacement of the muscle belly in relation to the metatarsus that are undergone by the intrinsic muscles. Initially, the feet of both species exhibit the primordia of four dorsal and four ventral muscles (figure 2ab). The most lateral primordia of the dorsal and ventral sides of the foot generate the EBDIV and ABDIV, respectively. Both muscles become well developed by HH36 in quail embryos. Nevertheless, in budgerigar embryos, EBDIV degenerates soon after it separates from the other muscles, and is reduced to a thread at HH37 (the presence of a small EBDIV has been reported for one species of parrot (Berman 1984), but the muscle is absent in other described Psittaciformes (Steinbacher 1935, Carril et

al. 2014)). On the other hand, the ABDIV becomes strongly developed and its muscle belly extends for four fifths of the metatarsal length (figure 3A).

These differences in muscle size could generate an asymmetric relation of forces, which is coherent with the rotation of dIV observed at HH35. The absence of a muscle inserted in the medial side and a stout muscle in the lateral side could account for the rotation of dIV from medial to lateral. Then, as the lateral side of the phalanges passes to face ventral, the action of ABDIV results in flexion of the digit, which acquires a right angle in relation to the limb main axis (HH37, figure 3bd). Further flexion of dIV by action of ABDIV, while being constrained by the extensor muscles, would lead to the final zygodactyl orientation, attained at HH38.

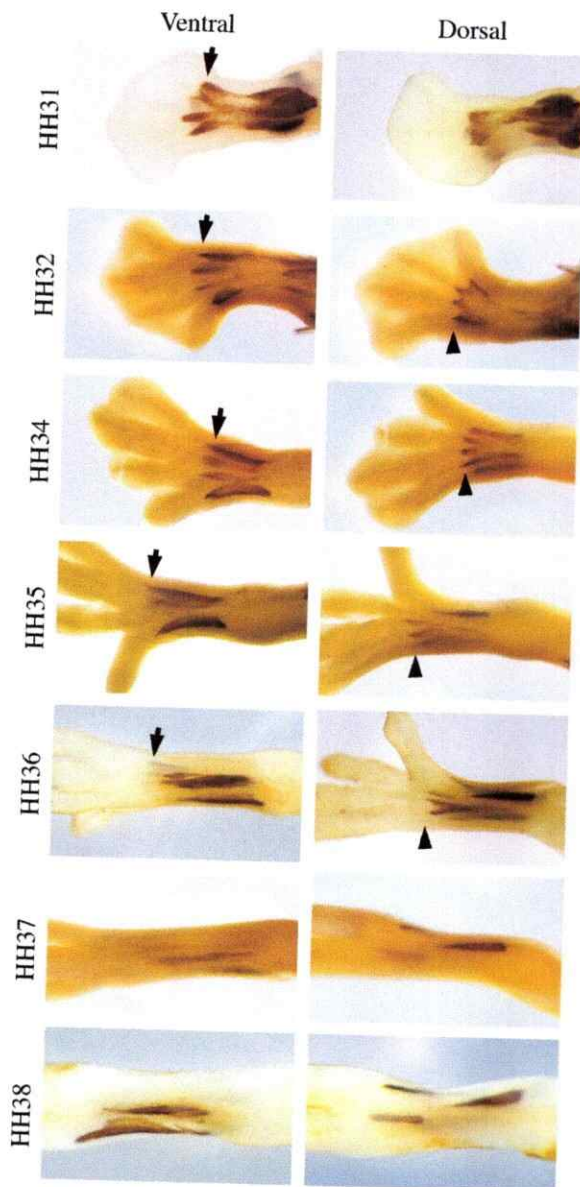
**(c) Muscular Paralysis in Budgerigar results in anisodactyl feet**

To test the hypothesis of the influence of early muscular activity on the development of zygodactyly, we pharmacologically immobilized budgerigar embryos and examined the resulting foot phenotype. The *in ovo* injection of the cholinergic agonist and neuromuscular blocker Decamethonium bromide (n=8) produced paralysis in budgerigar embryos. Treated embryos were anisodactyl at HH37 and HH38, and did not develop the *TrA* (Figure 3c).

**(d) Both EBDIV and ABDIV are lost in the development of the zebrafinch (Passeriformes)**



Most foot muscles are known to have been lost or vestigialized in Passeriformes (Hudson 1937, Raikow 1987), but their early development is still unknown. Taking into account the influence of muscle action on the development of zygodactyly in the budgerigar, we investigated the



**Figure 3.4:** Immunohistochemical reaction against myosin 2 showing the early development of dorsal and ventral foot muscles in zebra finch. No muscles disappear at HH37. Arrows: EBDIV; Arrow heads: ABDIV.

development of hind limb muscles in the anisodactyl foot of the zebra finch (*Taenopygia gutatta*). We observed that, similar to quails and budgerigars, the primordia of four ventral and four dorsal muscles are present at early stages (figure 4). However, most intrinsic muscles become vestigial during development and are almost indiscernible at HH37 (figure 4). The absence of both EBDIV and ABDIV acquires special significance since phylogenetic analyses of fossil and molecular data suggest that Passeriformes could have evolved from zygodactyl ancestors and are thus secondarily anisodactyl (see discussion below).



### 3.4 Discussion

Whole-mount immunostaining of the small developing muscles and tendons allowed us to compare the embryonic musculoskeletal system of anisodactyl and zygodactyl birds. These observations revealed that changes in the orientation of budgerigar dIV during ontogenesis occur concomitant to the modifications undergone by the intrinsic muscles controlling it. While the anisodactyl quail develops fully functional ABDIV and EBDIV, the zygodactyl budgerigar fails to develop an EBDIV. In the absence of the EBDIV restricting dIV movements, the abduction of dIV could cause the ontogenetic transition from anisodactyly to zygodactyly. Consistently, paralyzed budgerigar embryos develop an anisodactyl foot, which confirms that muscle activity is necessary for the ontogenetic re-orientation of dIV. In summary, our new data provides compelling evidence that the development of the zygodactyl foot in the budgerigar is caused by the asymmetric action of intrinsic muscles controlling dIV.

Since the strong extrinsic flexor and extensor muscles can control more than one digit, most studies of zygodactyl foot have looked for a common muscular organization of the extrinsic muscles controlling both retroverted digits dI and dIV in zygodactyl birds (Bock and Miller 1959, Raikow 1985). Our

investigation of the ontogenesis of the musculoskeletal system identifies the reduction of small intrinsic muscles simultaneous to digit retroversion as the most likely cause for the development of zygodactyly in the budgerigar. The identification of the importance of intrinsic muscles brings an important new aspect to consider in the evolution of specializations in the avian foot.

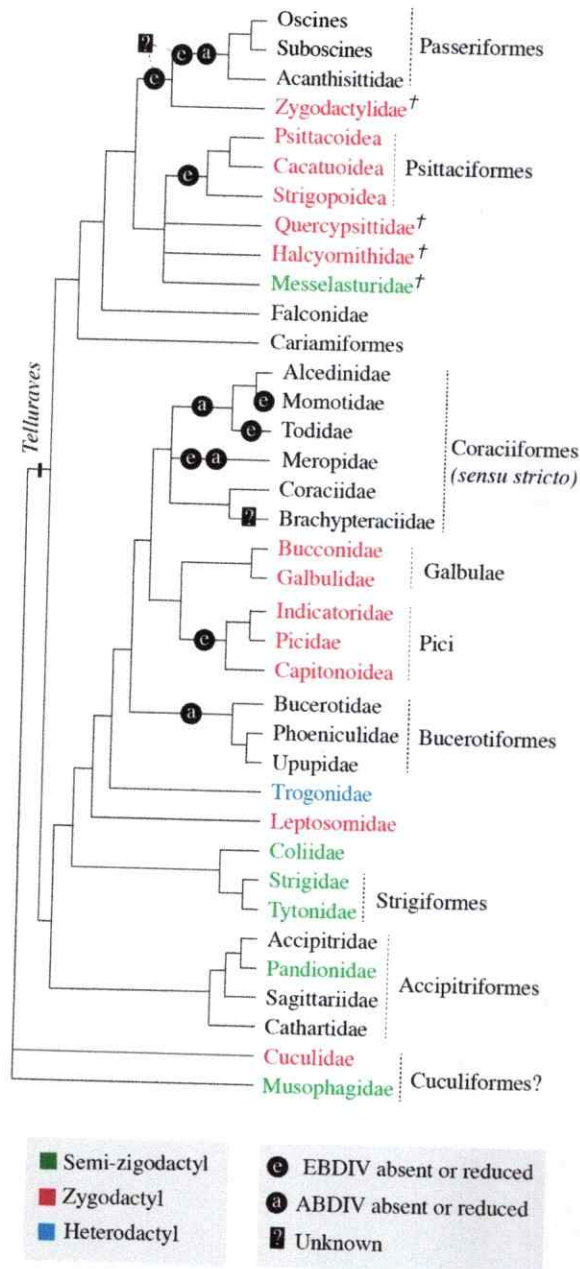
#### **(a) Epigenesis and Convergence**

The development of the musculoskeletal system depends on the functional interlocking of initially independent processes. Muscles, tendons and bones have different embryological origins. When brought together, their interactions have reciprocal morphogenetic effects (Schweitzer et al. 2010). Consequently, those interactions are potential sources of variation. Modifications in the early muscle precursor tissue can cause variations in the skeleton, and vice-versa. The developmental mechanism here proposed for the origin of zygodactyly provides an example of the power of those embryonic interactions to generate and drive evolutionary transformations.

The avian tarsometatarsus originates from the fusion among the diaphyses of metatarsals II, III, and IV and the distal tarsal cartilage (Namba et al. 2010). This fusion initially produces a common ossified diaphysis with three independent cartilaginous epiphyses in each side. At the proximal end, the interaction with tendons models the hypotarsus. At the distal end, the complex

combination of muscles controlling the digits results in an intricate set of epigenetic influences over each of the individual epiphyses. For example, birds which lack intrinsic muscles of the foot usually present small *incisuras intertrochlearis*, narrow and parallel trochleae, etc. We propose that zygodactyly and the associate *TrA* are produced by this same kind of process. They result from the epigenetic influence of muscular forces over the skeleton.

The reoccurrence of a similar set of influences over the skeleton of related lineages could cause convergent evolution (Losos et al. 2000, Huang et al. 2013, Machado-Schiaffino et al. 2014). The early presence of the primordia of intrinsic muscles of the foot and its variable posterior disappearance or reduction yield a drive for the repetition of similar influences and, consequently, for the transformation of the avian foot skeleton. These two factors – the effect of muscle over the form of digits *plus* the bias for the transformation of intrinsic muscles – provide a mechanistic explanation for the convergent evolution of zygodactyly. The convergence would have been facilitated by the flexibility of muscular system added to its influence over the skeleton. As muscles were reduced, lost or reacquired, similar skeletal morphologies evolved in parallel, including zygodactyly.



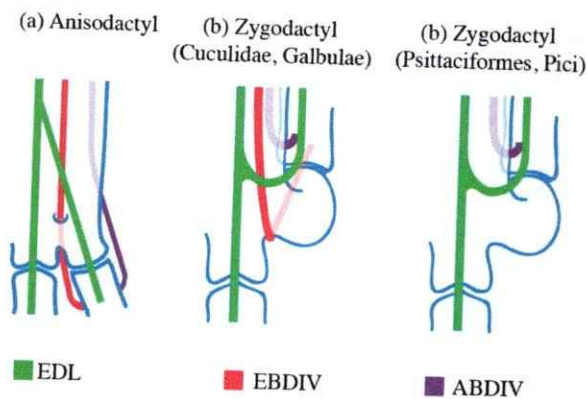
**Figure 3.5.** Phylogeny of Telluraves plus Cuculidae and Musophagidae depicting the position of Zygodactylidae and the loss of the intrinsic muscles of dIV. Clades depicted were congruently obtained by (Hackett et al. 2008, Kimball et al. 2013, McCormack et al. 2013). The position of Zygodactylidae and stem Psittaciformes follow (Mayr 2008, DeBee 2012, Mayr et al. 2013).

(b) **Zygodactyly in extant and fossil birds**

Zygodactyly has originated at least three times in extant birds: in Piciformes (Pici + Galbulae), Psittaciformes, and Cuculidae (figure 5). The Cucko-roller (*Leptosomus discolor*) may represent a fourth independent origin (Forbes-Watson 1967, Goodman 2001, Kirchman et al. 2001). The developmental mechanism proposed for the origin of zygodactyly – an unbalance of forces in the lateral and medial sides of the proximal phalanx – may also explain the evolution of zygodactyly in Pici (barbets, toucans, woodpeckers and allies). They are the only other Telluraves (clade comprising most arboreal birds) – besides parrots – that have lost EBDIV but conserve ABDIV (figure 5) [14, 15].

Extant Galbulae (Swierczewski and Raikow 1981) and Cuculidae (Berger 1952, Berger 1953, Berger 1960) exhibit the EBDIV, and a furrow (*sulcus extensorius*) on the dorsal tarsometatarsus indicates that EBDIV is also present in stem Cuculidae (Mayr 2006, Mourer-Chauviré et al. 2013), stem Psittaciformes (Mayr 2002, Mayr 2011, Ksepka and Clarke 2012, Mayr et al. 2013), and stem Piciformes (Mayr 2009, Mayr and Gregorová 2012). Those taxa have in common two characteristics: a *TrA* smaller than the one present in crown Psittaciformes and crown Pici (Olson 1983, Kirchman et al. 2001, Mayr 2006, Mayr 2009, Mayr 2011, Mayr and Gregorová 2012, Mourer-Chauviré et al.

2013) and an unusual trajectory of the EBDIV tendon: it does not pass through a foramen between mtIII and mtIV – the *canalis interosseus distalis* – as in most anisodactyl birds; it goes over the *incisura intertrochlearis lateralis* and backward to its insertion on the medial side of the proximal phalanx (figure 6) (Steinbacher 1935, Mayr 2002, Mayr 2003, Mayr 2006, Mayr and Gregorová 2012).



**Figure 3.6.** The topology of dIV tendons in (a) anisodactyl (b), zygodactyl with EBDIV, (c) and zygodactyl lacking the EBDIV. In (b) the EBDIV does not pass through the *canalis interosseus distalis*. In (c) the tendon is absent. Modified from (Steinbacher 1935).

If a mechanism similar to the one proposed here for crown Psittaciformes and crown Pici is responsible for the development of zygodactyly in other taxa, an unbalance of forces acting on the proximal phalanx of dIV may be caused by subtler dissimilarities, like differences in the size of the muscles or differences in the time and position of insertion of the

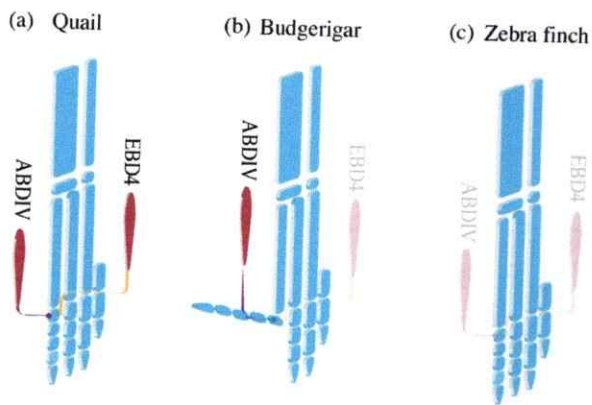
tendons, as suggested by the unusual trajectory of the EBDIV tendon over the *incisura intertrochlearis lateralis* (not through the *canalis interosseus distalis*).

The fossil record also shows that the condition observed in crown Psittaciformes and crown Pici (absence of the EBDIV, and large *TrA*) is convergently derived from zygodactyl ancestors having a smaller *TrA* and the tendon of EBDIV passing over the *incisura intertrochlearis distalis*. This morphological pattern suggests that the form and size of the *TrA* are related to the degree of unbalance of the forces acting over the metatarsus during development: those birds who lost or vestigialized EBDIV – like crown Pici, crown Psittaciformes and, probably, Zygodactylidae (see above) – exhibit larger *TrA* than those taxa that keep a EBDIV, like Galbulae and Cuculidae.

#### (c) Zygodactylidae and the Loss of ABDIV in Passeriformes

The presence of a *TrA* in the distal metatarsal of dIV allows the identification of zygodactyly even in non-articulated fossil skeletons. A group of those fossils has been recognized as a family of extinct birds appropriately called Zygodactylidae (Mayr 2009, DeBee 2012). Even though they exhibit a well developed *TrA* in the metatarsus IV, similar to that found in Pici and Psittaciformes, cladistic analysis points that Zygodactylidae is the sister taxon of passerines (Passeriformes) (figure 5) (Mayr 2008). On the other hand, one of the most robust data produced by the new avian molecular phylogenies is the

sister relationship between Passeriformes and Psittaciformes (Ericson et al. 2006, Hackett et al. 2008, Suh et al. 2011, Wang et al. 2012, Kimball et al. 2013, McCormack et al. 2013, Yuri et al. 2013). Therefore, both the extinct and extant outgroups to Passeriformes are zygodactyl, suggesting that Passeriformes had zygodactyl ancestors (Mayr 2009).



**Figure 3. 7.** Diagram illustrating the distribution of dIV intrinsic muscles in (a) the primary anisodactyl quail, (b) the zygodactyl budgerigar, and (c) the putative secondary anisodactyl zebra finch. Budgerigar lost the EBDIV but conserves the ABDIV. Both muscles are absent in the zebra finch. Muscles are absent in the red; skeleton in blue.

Considering our hypothesis on the role of ABDIV in the re-orientation of dIV in Psittaciformes, this muscle would be expected to have been present in Zygodactylidae. Therefore, the inferred ancestral state for the hypothetical clade (Psittaciformes (Zygodactylidae + Passeriformes)) would be the presence of ABDIV (figure 5). In this scenario, the further reduction of ABDIV in the lineage of passerine birds could have caused the loss of



zygodactyly, making them secondarily anisodactyl. Passeriformes would be anisodactyl not by an equilibrium of forces, as in primarily anisodactyl birds, but by the absence of any force acting on the lateral and medial sides of the proximal phalanx (figure 7), similar to paralyzed budgerigars. The presence of the primordia of the intrinsic muscles of the foot and their differential loss during the development of each taxon indicate the phylogenetic flexibility of the trait and supports this possibility.

#### (d) Heterodactyly

The singular arrangement known as heterodactyly is unique to birds of the family Trogonidae. Those birds have digits I and II reverted and digits III and IV pointing forward. Molecular phylogenies include trogons in the Telluraves assemblage (Hackett et al.), but its specific position is unresolved. If a similar mechanism of asymmetrical forces at early development is responsible for the unique change of digit II orientation found in trogons, the loss of muscles ought to have occurred in the opposite sides of that found in zygodactyl birds. The dorsal *Musculus abductor digitum 2* (ABDII) is inserted in the medial side of proximal phalange of the dII and the ventral *Musculus adductor digitum II* (ADDII) is inserted in the lateral side. As the toe must rotate in the opposable direction in relation to the zygodactyl feet, the rotation would demand the loss of the ventral intrinsic muscle (ADDII). The hindlimb

muscles of two species of the genus *Trogon* have been described by Maurer and Raikow (Maurer and Raikow 1981). The species described present the ABDII but do not have the ADDII, suggesting that a similar mechanism of asymmetric muscular action could be responsible for the development of heterodactyly. Furthermore, they are the only Telluraves clade that have lost ADDII but not ABDII, since other clades that do not have ADDII also lost ABDII (some Coraciiformes, Upupiformes, Pici, Psittaciformes and Passeriformes) (Maurer and Raikow, Swierczewski and Raikow, Raikow).

### 3.5 Conclusion

Extant birds exhibit a diversity of foot forms, which is absent in any other archosaur group. The mechanism here proposed for the origin of zygodactyly is based on transformations that indirectly allowed that diversification in Aves. While the intrinsic muscles of the foot are relatively small and homogeneous in crocodylians and theropod dinosaurs, birds have larger, more variable and individualized muscles (Hutchinson 2002), which allowed their differential influence on the skeleton. Furthermore, zygodactyl birds are very altricial and maintain the skeleton cartilaginous for most of the embryonic period. We propose that foot muscle diversity and their action over the flexible embryonic skeleton caused the appearance of new foot configurations in birds, like zygodactyly. New fossil discoveries and further investigations on the anatomy and, specially, the development of the foot of other zygodactyl birds will contribute to test this hypothesis and further clarify the relationship between foot morphology and muscle development.

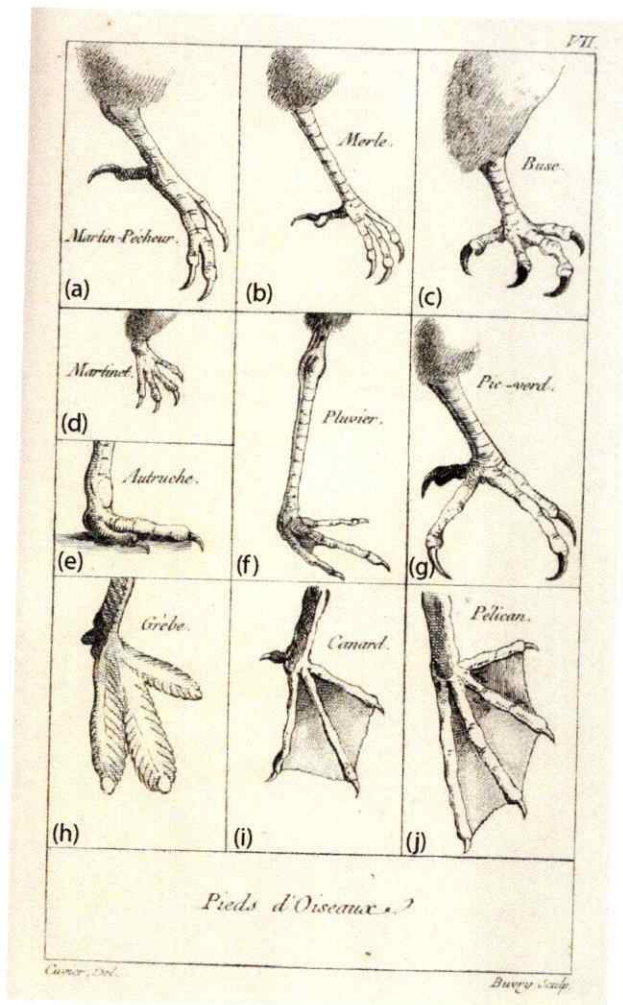
## The Precocial-Altricial Spectrum and the Evolution of Specialized Feet in Birds

**Abstract:** Specialized morphologies of bird feet have evolved several times independently as different groups have become palmate, totipalmate, zygodactyl, semi-zygodactyl, or syndactyl. Birds have also convergently evolved similar modes of development, in a spectrum that goes from precocial to altricial. We compared the morphology of feet and modes of development of all extant avian families. We found that variations in the arrangement of toes in respect to the ancestral condition have occurred only in altricial groups. Those groups represent four independent events of super-altriciality and at least ten independent transformations of toe arrangement (four zygodactyl, three semi-zygodactyl, one heterodactyl and one pamprodactyl group). Syndactyly also evolved independently in several super-altricial taxa. We propose the correlation between foot morphology and mode of development is related to skeletal heterochronies due to altriciality and epigenetic influences of muscular activity over the developing foot.

**Keywords:** altricial, heterodactyl, pamprodactyl, precocial, syndactyl, zygodactyl.

## 1. Introduction

Many of the locomotor adaptations that allow extant birds to explore different niches occur in the feet. Modifications of the feet allow birds to be fast runners, to climb and perch trees, to swim under and above the water surface, to hunt and fish, and to walk in the mud and over aquatic vegetation, among other abilities. The great variety of forms generated by those modifications was employed in early avian classifications (Linnaeus 1758, Brisson 1760, Lacépède 1799, Cuvier 1836). The anatomy of the foot has been described in seven main types: **Anisodactyl** feet have digit II (dII), digit III (dIII) and digit IV (dIV) pointing forward and digit I (dI) pointing backward (figure 1b and c). From the basal anisodactyl condition three feet types have arisen by modifications in the orientation of digits. **Zygodactyl** feet have dI and dIV oriented backward and dII and dIII oriented forward (figure 1g), a condition similar to **Heterodactyl** feet, which have dI and dII oriented backward and dIII and dIV oriented forward. **Pamprodactyl** feet have all four digits pointing forward (figure 1d). Other feet modifications involve the presence of interdigital webs. **Palmate** feet have webs between dII, dIII, and dIV (figure 1i) and **Totipalmate** feet have an additional membrane connecting dI and dII (figure 1j). Finally, the partial fusion of two or more digits produces **Syndactyl** feet (figure 1a).

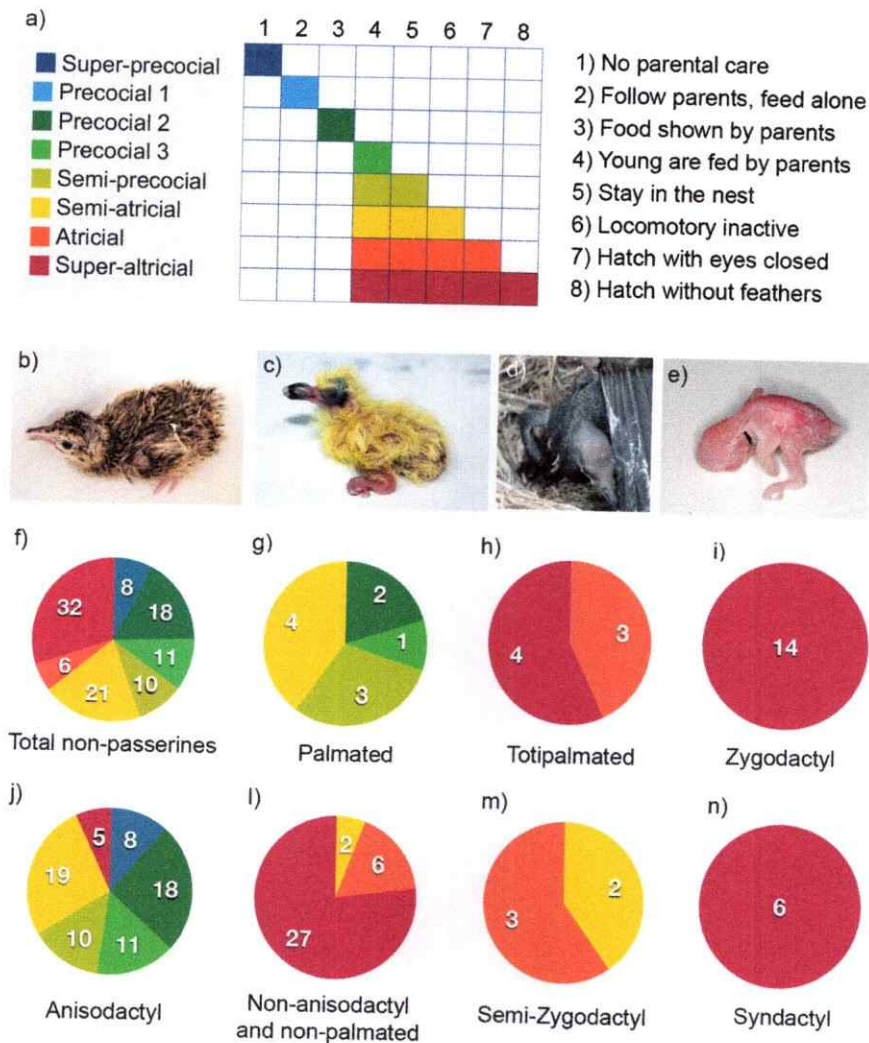


**Figure 4.1. The diversity of foot morphology was the base of early avian systematics.** Illustration of bird foot diversity in Cuvier *Le Reine Animal* (Cuvier 1836): (a) the syndactyl kingfisher; (b) the anisodactyl thrush and (c) buzzard; (d) pamprodactyl swift; (e) bidactyl ostrich; (f) a semi-palmate plover; (g) a zygodactyl woodpecker; (h) a lobate grebe; (i) a palmate duck; (j) a totipalmate pelican.

Birds have also been classified according to their morphology upon hatching and the level of parental care thereafter, in a spectrum that varies from super-precocial to super-altricial (figure 2a) (Nice 1962, Starck 1993, Starck and Ricklefs 1998). **Super-precocial** birds lack any parental care, a condition exclusively found in the family Megapodiidae (megapodes). **Precocial** birds show active locomotion at hatching and follow their parents (figure 2b);

this is considered to be the ancestral condition to Neornithes (Tullberg et al. 2002, Birchard et al. 2013). They have been sub-divided in accordance to their parental dependence for feeding: Precocial 1 (P1) feed on their own; Precocial 2 (P2) has the food shown to them by their parents; Precocial 3 (P3) are fed by their parents. **Semi-precocial** birds show active locomotion but are fed in the nest and leave it only in case of danger. Birds in the altricial spectrum hatch with poor motor activity and are totally dependent on their parents. They are locomotory inactive and stay in the nest for long periods. Altricial birds can be subdivided in **semi-altricial**, **altricial** and **super-altricial** following morphological characteristic of hatchlings. Semi-altricial birds hatch with feathers and eyes open, altricial birds hatch with feathers, but eyes closed (figure 2c), and super-altricial birds hatch without feathers and eyes closed (figure 2d and e).

In the last years, molecular phylogenies employing large taxon sampling have robustly established some high-level avian relationships (Ericson et al. 2006, Hackett et al. 2008, Kimball et al. 2013, McCormack et al. 2013, Yuri et al. 2013). The results show that the evolutionary history of both traits – foot morphology and developmental mode – is very homoplastic. The altricial mode of development has evolved independently in at least four avian clades: Strisores (nightjars, swifts, hummingbirds and allies) (Mayr 2010),



**Figure 4.2. The diversity of developmental modes and feet morphologies in extant birds.** (a) The mode of development is classified in a spectrum that goes from super-precocial to super-atricial following behavioural and morphological characteristics; (b) *Nothoprocta perdicaria* (Tinamidae), a precocial new-hatching; (c) *Columba livia* (Columbidae), an altricial new-hatching; (d) *Phalacrocorax brasilianus* (Phalacrocoracidae), a super-atricial “water-bird” new-hatching; (e) *Melopsittacus undulatus* (Psittacidae), a super-atricial “land-bird” new-hatching; (f) The distribution of developmental modes in non-passerines families of extant birds (g); The developmental modes in palmate families of extant birds; (h) The developmental modes of totipalmate families of extant birds; (i); All zygodactyl families are super-atricial (j); The distribution of developmental modes in non-passerines anisodactyl families (l); The distribution of developmental modes in non-passerines and non-anisodactyl families (m); Semi-zygodactyl families are altricial or semi-atricial and (n); All syndactyl families are super-atricial.



Cuculiformes (cuckoos), Aequornithes (the “water bird” assemblage) (Mayr 2011), and Telluraves (the “land bird” assemblage)(Yuri et al. 2013) (figure 3). Most variations in the morphology of foot – palmate and totipalmate feet, syndactyly, zygodactyly and semi-zygodactyly – have also evolved convergently in different families.

Examining the phylogenetic distribution of foot morphology and mode of development, we found a correlation between variations in the arrangement of toes and the altricial mode of development. Considering the influence of altriciality in the rates of skeletal growth and the action of foot muscles at early development, we propose a developmental mechanism to explain this correlation.

#### 4.2. Material and Methods

The nomenclature employed for avian families follow the International Onithological Comittee (IOC) world bird list 4.2. Each of the 107 non-passerines families was classified by its foot morphology and mode of the development, Passeriformes comprise more than half of total bird families and are uniforms in relation to both traits. The mode of development classification follows Nice (Nice 1962) and Starck (1993), except that for sake of clarity altricial 1 are called altricial, and altricial 2 are called super-altricial. Foot morphology follows descriptions in Raikow (Raikow 1985). Information about both traits was complemented by specialized literature. The phylogenetic history of each trait was traced by parsimony reconstruction employing Mesquite software Version 2.75 (Maddison and Maddison 2011). Embryo from the collection in the Laboratory of Ontogeny and Phylogeny in the University of Chile were photographed in an Olympus stereoscopic microscope.

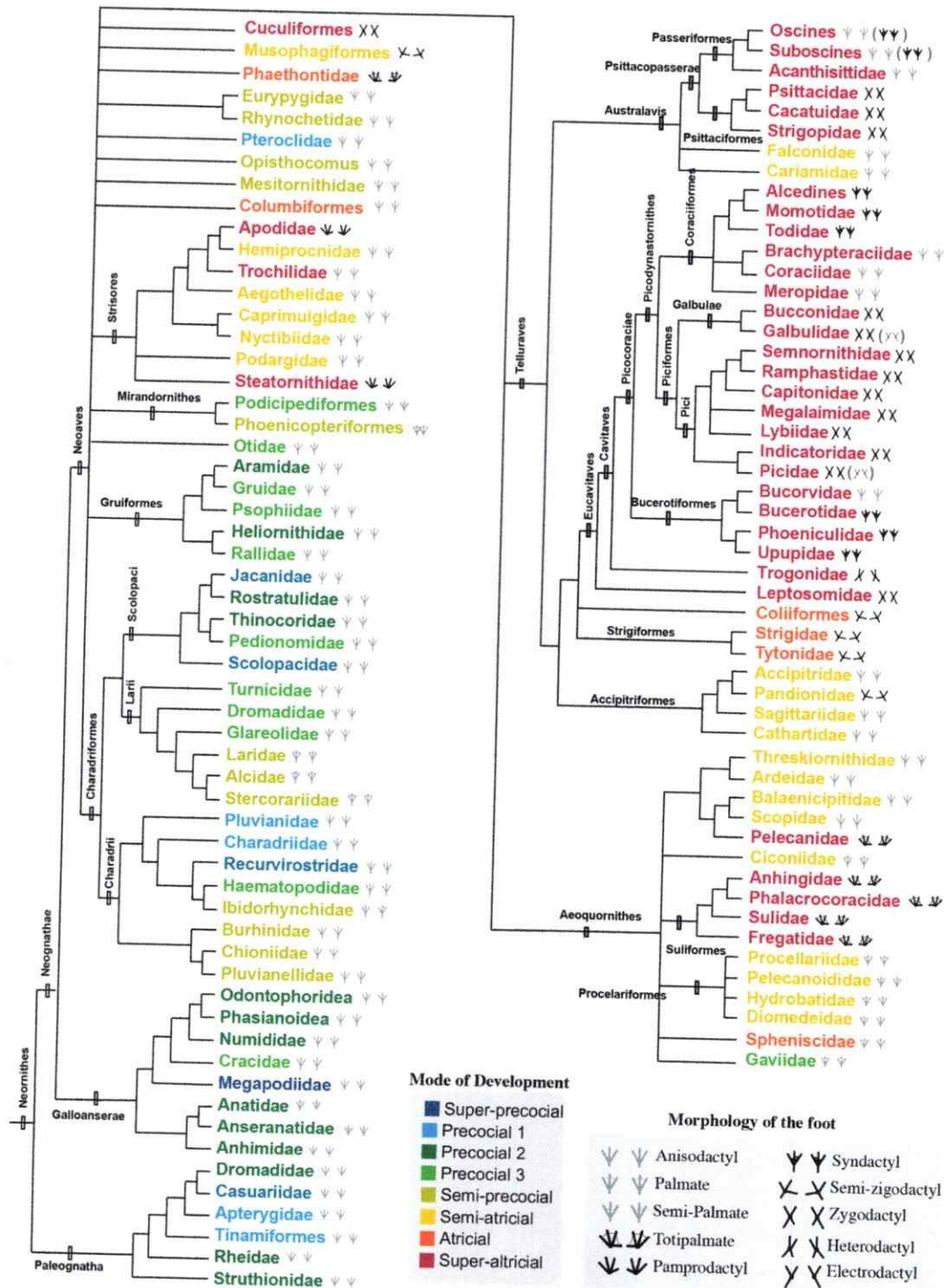
### 4.3. Results

#### (a) Precocial birds are anisodactyl or palmated

The inferred ancestral mode of development for Neornithes is P2 (Figure 4). Ratites and tinamous (Paleognathae) are P1 or P2; Ducks and allies (Anseriformes) are P2, while pheasants and allies (Galliformes) are Super-P, P2 or P3. Many Neoaves are also precocial: Sandgrouses (Pteroclididae) are P1; shorebirds (Charadriiformes) could be P1, P2, P3 or Semi-P; Gruiformes *sensu stricto* are P2; loons (Gaviidae) and grebes (Podicipedidae) are P3. The Semi-P mode of development evolved in kagu (Rhynochetidae), sunbittern (Eurypygidae), mesites (Mesitornithidae), and hoatzin (Opisthocomidae). All precocial and semi-precocial birds are anisodactyl or palmate (figure 2g and 2j). Palmated feet have evolved independently in ducks (Anatidae), gulls and allies (Larii), flamingos (Phoenicopteridae), and grebes (Gaviidae). Some precocial birds have webs only between proximal phalanges (Anseranatidae) or lobate webs (Heliornithidae, Podicipedidae, and some Rallidae) (figure 1H).

#### (b) Totipalmate birds are altricial or super-altricial

Totipalmate feet are present in tropicbirds (Phaethontidae), pelicans (Pelecanidae), gannets (Sulidae), frigatebirds (Fregatidae), cormorants (Phalacrocoracidae), and darters (Anhingidae). Tropicbirds are altricial, but all other totipalmate families are super-altricial (figures 2h and 3).



**Figure 4.3. The phylogenetic distribution of development modes and feet morphologies.** A coherent phylogeny based on (Ericson et al. 2006, Hackett et al. 2008, Kimball et al. 2013, McCormack et al. 2013, Yuri et al. 2013) of all extant bird families depicting the mode of development and the morphology of foot.

Totipalmatè birds have been historically classified in the order Pelecaniformes (Wetmore 1934). However, the totipalmate foot is now thought to have evolved independently in three clades: Pelecanidae, Suliformes (Sulidae, Fregatidae, Phalacrocoracidae, and Anhingidae), and Phaethontidae (Ericson et al. 2006; Hackett et al. 2008, Kimball et al. 2013, McCormack et al. 2013, Yuri et al. 2013). Pelicans are closely allied to the semi-altricial and anisodactyl shoebill (Balaenicipitidae) and hamerkop (Scopidae), the three of them being grouped in the sister taxon of a clade composed by herons (Ardeidae) and ibis (Threskiornithidae). Both Pelecanidae and Suliformes belong to the Aequornithes clade (figure 3). The phylogenetic position of Phaethontidae is uncertain (Hackett et al. 2008, McCormack et al. 2013, Yuri et al. 2013), but they can also be included within Aequornithes (Cracraft et al. 2004, Livezey and Zusi 2007).

Other Aequornithes with webbed feet are the penguins (Spheniscidae) and the Procellariiformes (Procellariidae, Diomedidae, Hydrobatidae, and Pelecanoididae). Molecular phylogeny supports Spheniscidae as the sister group of Procellariiformes (Hackett et al. 2008, Kimball et al. 2013, McCormack et al. 2013, Yuri et al. 2013). Spheniscidae are altricial and Procellariiformes are semi-altricial. The two taxa are characterized by a highly reduced hallux (or totally absent in the case of albatrosses) and it is

difficult to discern if they have a web between dI and dII, so if they are palmate or totipalmate.

**(c) Heterodactyl and Zygodactyl birds are super-altricial**

All zygodactyl and heterodactyl birds are super-altricial (figure 2i). Trogonis (Trogonidae) are the only extant heterodactyl birds. Zygodactyl feet are present in cuckoos (Cuculidae), *Leptosomus* (Leptosomidae), parrots (Psittaciformes), and woodpeckers, toucans, barbets, jacamars and allies (Piciformes) (figure 3). *Leptosomus* is sometimes described as semi-zygodactyl (Forbes 1880), but modern observations of live nestling and adults show that it is fully zygodactyl, even when not perched (Forbes-Watson 1967, Goodman 2001).

Zygodactyl feet have evolved independently in different super-altricial taxa (figure 4). Cuckoos (Cuculidae) are congruently supported within Neoaves, but unrelated to other super-altricial birds (Ericson et al. 2006, Hackett et al. 2008, Kimball et al. 2013, McCormack et al. 2013, Yuri et al. 2013). All other zygodactyl birds (Leptosomidae, Piciformes, and Psittaciformes) are Telluraves (Hackett et al. 2008, Kimball et al. 2013, McCormack et al. 2013, Yuri et al. 2013), a clade divided in two main branches: Australavis and Afroaves (Ericson 2012). Basal families of both clades are semi-altricial, implying that super-altriciality probably evolved twice in Telluraves

(figure 4). The zygodactyl parrots pertain to Australavis, forming Psittacopasserae (Suh et al. 2011) with Passeriformes (Figure 3). A zygodactyl extinct family, called Zygodactylidae, is considered sister group of Passeriformes (Mayr 2008). *Leptosomus* and Piciformes are Afroaves (Hackett et al. 2008, Kimball et al. 2013, Yuri et al. 2013). *Leptosomus* is a basal Eucavitaves, group that contains Piciformes as the sister taxa of Coraciiformes (figure 3) (Kimball et al. 2013): Zygodactyly is inferred to have evolved independently in both groups – *Leptosomus* and Piciformes. Non-zygodactyl fossil representatives of stem Cuculidae (Baird and Vickers-Rich 1997, Mayr 2006, Mourer-Chauviré et al. 2013), Leptosomidae (Mayr 2008), Psittaciformes (Mayr et al. 2010, Mayr 2011, Ksepka and Clarke 2012, Mayr et al. 2013), Piciformes (Mayr 2009) and Pici (Mayr and Gregorová 2012) are known. Morphological differences (Olson 1983, Raikow and Cracraft 1983) and the existence of non-zygodactyl stem Pici representatives (Mayr and Gregorová 2012) suggest the possibility that Pici and Galbulae are independently zygodactyl. Therefore, zygodactyly has evolved four or five times in extant birds associated to three independent origins of super-altriciality: The heterodactyl trogons (trogonidae) are the sister taxa of Picocoraciae (Mayr 2011) and, as all Eucavitaves, are super-altricial (Figure 3).

(d) Semi-zygodactyl birds are altricial or semi-altricial

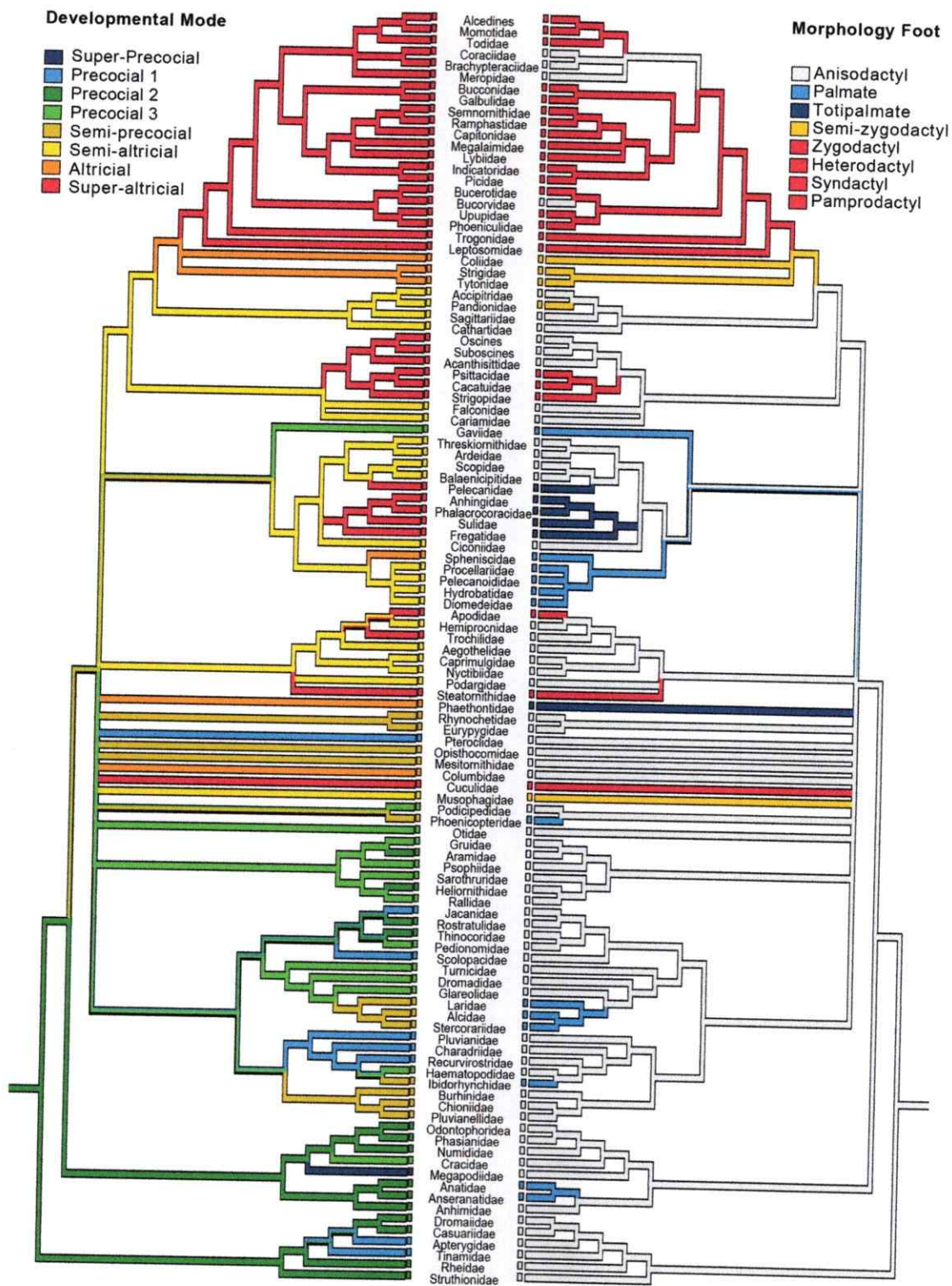
Semi-zygodactyl birds are able to facultative abduct dIV, reaching a zygodactyl or semi-zygodactyl position. Turacos (Musophagidae), ospreys (Pandionidae), owls (Strigiformes), and mousebirds (Coliidae) are semi-zygodactyl. Musophagidae and Pandionidae are semi-altricial, whereas Strigiformes and Coliidae are altricial (figure 3).

Semi-zygodactyly evolved independently at least three times in extant birds (figure 4). Turacos (Musophagidae) are semi-altricial Neoaves, and have been historically classified as the sister group of Cuculidae, but their relationship remains uncertain. Other extant semi-zygodactyl birds are basal Afroaves (Ericson 2012). Ospreys (Pandionidae) are Acciptriformes. The altricial owls (Strigidae and Tytonidae) and mousebirds (Coliidae) are closely related to Eucavitaves. The exact position of mousebirds is controversial and they can be the sister taxa of owls. Therefore, semi-zygodactyly in extant birds is inferred to have evolved in two independently semi-altricial clades and one or two altricial clades, depending on the phylogenetic position of Coliidae. Some raptorial-like fossil stem Psittaciformes are also believed to have been semi-zygodactyl (Mayr et al. 2010, Mayr et al. 2013).

**(e) Pamprodactyl birds are super-altricial**

Swifts (Apodidae) represent the only uncontroversial pamprodactyl taxon. The parakeet genus *Micropsitta* (Psittaciformes) and the oilbird





**Figure 4.4. Character history of developmental modes and feet morphologies.** Character history traced by parsimony showing a correlation between multiples events of altriciality and foot morphology evolution. Passeriformes have been considered as three anisodactyl clades: Acanthisittidae, Suboscines, and Oscines. As discussed in the text, some Passeriformes, Meropidae and Trochilidae are syndactyl.

(Steatornithidae) are considered pamprodactyl by some authors, but their hallux seems to point medially, not forward (Collins 1983). They are all super-altricial. Mousebirds (Coliidae), on the other hand are altricial and facultative pamprodactyl.

Most Strisores families are semi-altricial and anisodactyl (figure 3). Super altriciality is inferred to have evolved twice: in Apodiformes (swifts and hummingbirds) and oilbirds (Steatornithes) (figure 4). Pamprodactyly in swifts and the peculiar morphology of oilbird foot evolved associated to those two independent origins of super-altriciality inside Strisores. Interestingly, the fossil *Eurofluvioviridaves* was first classified in Strisores (Mayr 2005), but later dismissed as *incertae sedis* based on the morphology of its zygodactyl feet (Nesbitt et al., 2011).

**(f) Syndactyl birds are super-altricial**

Syndactyly occurs as different grades of fusion of proximal phalanges of two or three toes in several passerine families (Passeriformes) [29, 30]; most kingfishers (Alcedines), todies (Todidae), motmots (Momotidae), bee-eaters (Meropidae), jacamars (Galbulidae), wood-hoopoes (Phoeniculidae), hoopoes (Upupidae), hornbills (Bucerotidae) [27, 29, 31], trogons (Trogonidae)

and some hummingbirds (Trochilidae) (Mayr 2003). The trait is highly plastic, but restricted to super-altricial birds (figure 2n). Except for Trochilidae, all syndactyl species are Telluraves. The trait could be synapomorphic for Cavitaves (Kimball et al. 2013), secondarily lost by taxa as Bucorvidae and some Alcedines (figures 3 and 4).

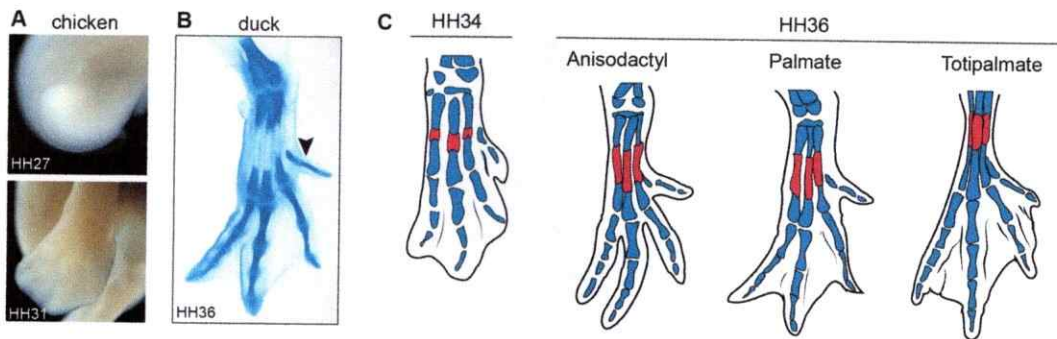
#### 4.4. Discussion

While the precocial spectrum is related mainly to behavioural variations, the altricial spectrum is characterized by morphological and ontogenetic differences, especially in rates of development. The phylogenetic pattern suggests a correlation between altriciality and two traits: (a) the presence of a web between dI and dII and (b) changes in the orientation of the toes. We discuss above the possible relation between altriciality and putative developmental process that could have influenced the evolution of those two traits.

##### (a) Totipalmate feet, developmental mode and hallux size.

Early embryos of birds have all digits connected by mesenchymal tissue, and the separation of digits begins at embryonic stage HH34 and is finished at HH36 (between embryonic days 8 and 10) in the domestic chicken (Hamburger and Hamilton 1951). The webs between digits disappear as the mesenchymal cells die by apoptosis in a distal to proximal direction (figure 5a). (Ede 1973). Therefore, the independent evolution of interdigital webs could be explained by the loss of apoptosis between dII, dIII, and dIV in different clades. The existence of semi-palmate feet (e.g. Anseranatidae) could be explained by the interruption of apoptosis in the distal-to-proximal direction.

Nevertheless, apoptosis between dI and dII does occur in palmate and semi-palmate feet. The evolution of totipalmate feet means the further absence of apoptosis between dI and dII in some clades, implying the existence of a possible antero-posterior direction of apoptosis, as suggested by some studies in chicken embryos (Bastida et al. 2004) (figure 5c).



**Figure 4.5. Differences in the apoptotic pattern of interdigital webs cause the differences seen in the adult morphology of bird feet:** (a) HH27 and HH31 chicken (*Gallus gallus*) limb bud showing the development of digits and the presence of interdigital membranes; (b) HH36 ducks (*A. platyrhynchos*), retain membranes between digits II, III and IV, losing only the web between digits I and II. Note as embryonic digit I is not so smaller than other digits as it is in adult ducks (black arrowhead); (c) The apoptosis of interdigital webs in embryonic feet after HH34 determines if a foot is either anisodactyl, palmate, or totipalmate.

The phylogenetic evidence for a correlation between the altricial mode of development and the absence of apoptosis of the web between dI and dII is weak. With the exception of the Phaethontidae, whose relationships are uncertain, all other totipalmate birds are Aequornithes, and the presence of totipalmate feet, although not a synapomorphy of this clade, could still bare

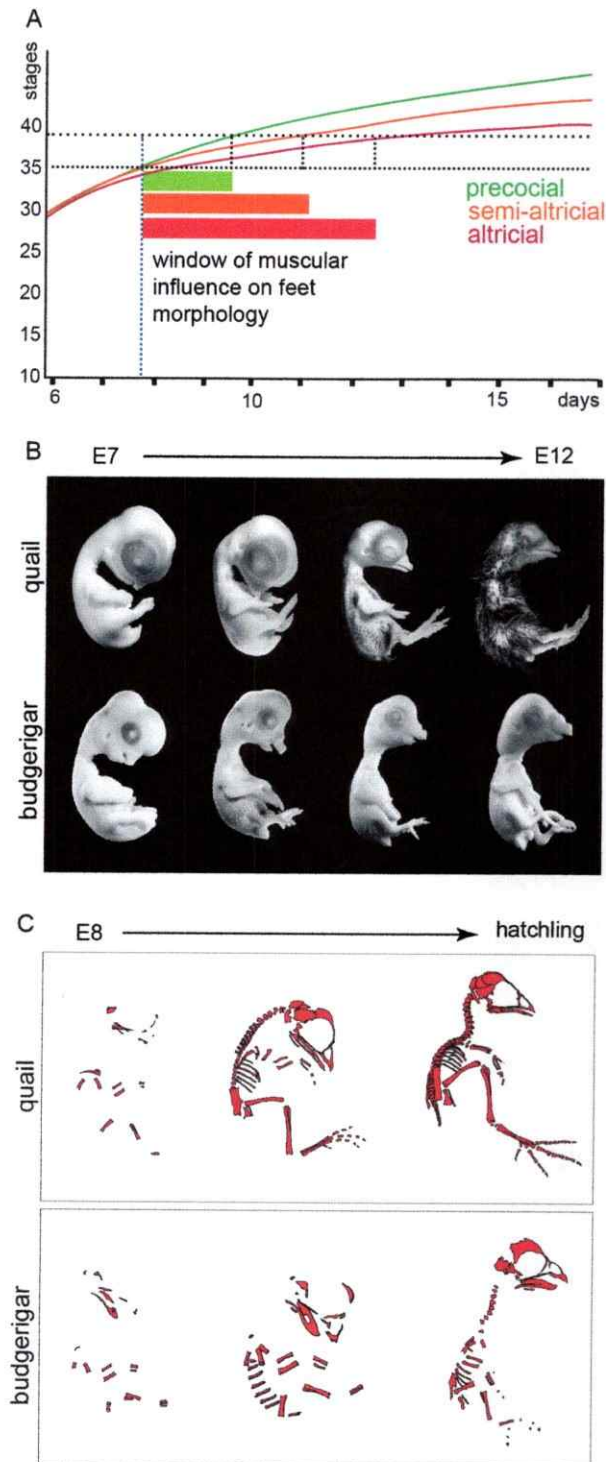
phylogenetic significance. Moreover, it has been pointed that all totipalmate Aequornithes have large halluces and the maintenance of the web could be related to its larger size, compared to most palmate birds which have a small hallux and, consequently, small webs that could be lost much easier than in totipalmate birds (Mayr 2005).

Nevertheless, it is notorious that precocial birds are never totipalmate – including the palmate Gaviidae, the only precocial Aequornithes – and pelicans are super-altricial totipalmate inside a clade composed by semi-altricial anisodactyl birds. Moreover, palmate birds usually have a small hallux as adults, but the difference is less prominent at early development when web apoptosis occurs (figure 5b). The question if developmental mode, hallux size, phylogenetic relationship or other factors represent a constraint for the evolution of a web between dI and dII remains open.

#### **(b) Embryonic muscular activity and evolution of avian toe's orientation**

The phylogenetic pattern strongly suggests that modifications in the orientation of digits have been allowed by morphological variations associated to altriciality. A major characteristic of altricial birds is the slowed late embryonic development, followed by fast post-hatching grow rates (except for Aequornithes) (Starck 1993, Grady et al. 2014) (figure 6a). The differences are





**Figure 4.6. The altricial mode of development and its relation to skeletal flexibility.** (a) The slowed-down late development and its consequent delay in ossification result in a bigger window of possibility for muscle activity to modify the skeleton (modified from (Starck 1993)). (b) Comparison between the two contrasting developmental modes of the precocial and anisodactyl quail (*Coturnix japonica*) and the super-altricial and zygodactyl budgerigar (*Melopsittacus undulatus*); (c) Comparison of ossification progress during embryonic time between quail and budgerigar. Both species begin to ossify around the same stage. Upon hatching time, precocial birds are almost completely ossified, locomotory active and able to feed alone, while altricial birds are poorly ossified, locomotory inactive and unable to feed by themselves.

particularly remarkable in the skeleton. Precocial and altricial birds begin ossification at similar stages, around HH32. Nevertheless, when altricial species hatch, large parts of the skeleton are still cartilaginous, while precocial hatchlings have ossified most of their skeleton (Maurer and Raikow 1981) (figure 6c). The slowing down of skeletal differentiation in altricial birds begins around HH35, precisely the period when digit orientation is defined (figure 6b).

The musculoskeletal system controlling toes is functional from around the second half of the embryonic period (HH35), also at the period digits orientations are being defined. It has been shown that the development of zygodactyl feet in Psittaciformes is caused by the action of asymmetrical forces during development due to the loss of intrinsic muscle of the feet (Botelho et al. 2014). These intrinsic muscles individually control the extension, flexion, adduction or abduction of toes. During the early embryogenesis of budgerigars (Psittaciformes), the *musculus extensor brevis digiti IV* degenerates, and in the absence of this muscle, the *musculus abductor digiti IV* abduces the embryonic digit IV until reaching a zygodactyl orientation. The same asymmetric muscular morphology is present in Pici (Swierczewski and Raikow 1981). A similar asymmetry in the musculature of digit II (presence of



the *musculus abductor brevis digiti II* and absence of *musculus adductor brevis digiti II*) is exclusively seen in the heterodactyl trogons.

We propose that the correlation seen between altriciality and feet morphology could be explained considering that the action of foot muscles can modify the orientation and shape of skeletal elements in a more radical way when exerted over the embryonic skeleton of altricial birds. This could be because the development of embryonic cartilages is plastic during a specific stage or "window" that is more prolonged in the generally slowed-down development of the skeleton of altricial birds (Figure 6a). Intrinsic foot muscle organization is highly variable in extant birds (Hudson 1937, Hudson 1948, Berger 1960, George and Berger 1966, Maurer and Raikow 1981, Swierczewski and Raikow 1981, Raikow 1985, Raikow 1987). Missing or reduced muscles generate asymmetric forces over individual digits. These asymmetric forces can transform the orientation of the digits only if the skeleton upon which they are acting is plastic during enough time. Zygodactyly, heterodactyly, and pamprodactyly would have developed in every super-altricial clade where asymmetric forces were acting on the toes. In this model, a different kind of musculoskeletal topology may not be required for the transition from a semi-zygodactyl to fully zygodactyl foot, which may require just a change in the duration of the period of skeletal plasticity, as suggested by the fact that semi-

zygodactyl birds are not super-altricial, but altricial or semi-altricial (figures 2m, 3, 4 and 6a).

Another characteristic possibly related to super-altriciality and the loss of intrinsic muscles of the foot is syndactyly. The condition could be related to the reduction of movements in the proximal phalange, since *Upupiformes*, *Alcediniformes*, *Passeriformes* and *Trochilidae* have lost most of the intrinsic muscle of the foot, specially the abductors (Hudson 1937, Maurer and Raikow 1981, Swierczewski and Raikow 1981, Zusi and Bentz 1984, Raikow 1987). Non-syndactyl taxa closely related to syndactyl birds usually present shallow *incisures intertrochlearis lateralis* and small divarication angles between digits, already indicating the presence of modified muscles of the foot. Altricial birds that present symmetric intrinsic muscles of the foot, like pigeons, are perfectly anisodactyl (Cracraft 1971).

#### 4.5. Conclusion

Birds are paedomorphic dinosaurs (Bhullar et al. 2012) and many of their characteristics are believed to be due to the conservation of juvenile characteristics: the loss of teeth, large brains and eyes, fusion of bones, etc (O'Connor et al. 2011, Balanoff et al. 2013). We propose that super-altriciality is related to further heterochronic transformation of some extant bird taxa. These ontogenetic changes resulted in a delay of skeletal development, with prolonged stages that allow for plasticity of the embryonic skeleton, which in turn allowed the transformation of foot morphology by the embryonic muscular system. In summary, we propose that secondary transformations of bird foot morphology are driven by two major processes: (i) the occurrence and direction of interdigital tissue apoptosis, and (ii) the influence of intrinsic muscles of the foot over the orientation of digits in birds where altriciality has increased plasticity during skeletogenesis (figure 7).

## CONCLUSIÓN:

### 5. Fósiles, Evolución y Desarrollo

A pesar de que la explicación recapitulacionista hoy resulta inapropiada y anacrónica, la conservación de patrones tempranos seguidos por modificaciones tardías es un fenómeno que demanda la atención de la Biología Evolutiva actual (Gould 1977).

Las transformaciones evolutivas en la estructura esquelética pueden ocurrir por variaciones en la condrogénesis temprana y/o la condrogénesis tardía. La condrogénesis temprana define la topología general del esqueleto, al determinar el número, tamaño y posición de las condensaciones mesenquimales. En muchas instancias las variaciones evolutivas en la estructura esquelética de los miembros son causadas por modificaciones en la condrogénesis temprana, como por ejemplo, el número de dígitos presentes en el ala y en la pata de las aves (Parker 1888, Hinchliffe 2002). Las diferencias

causadas por modificaciones en la condrogénesis tardía se originan durante la diferenciación y el crecimiento del esqueleto. Muchas de las características que distinguen al esqueleto de las aves modernas del de sus ancestros resultan de modificaciones en la condrogénesis tardía. Algunos ejemplos son la fusión de los tarsales proximales a la tibia, la fusión de los tarsales distales a los metatarsos, y la re-orientación de los dígitos (Morse 1874, Namba et al. 2010). Es la conservación del patrón de condrogénesis temprana, seguido por modificaciones en la condrogénesis tardía lo que genera una aparente recapitulación de la filogenia en la ontogenia.

El estudio de las variaciones en la condrogénesis tardía como agente de la transformación evolutiva ha ganado atención especialmente por su capacidad de esclarecer problemas macroevolutivos, es decir, el origen de innovaciones evolutivas que diferencian a grandes grupos de organismos. Es el caso, por ejemplo, del origen del caparazón de las tortugas (Hirasawa et al. 2013), el ala de los murciélagos (Farnum et al. 2007) y el oído medio de los mamíferos (Luo 2011). La investigación de estos casos se enfrenta con las dificultades asociadas al trabajo con organismos no modelós y con estructuras anatómicamente complejas, pero se beneficia de la integración de la morfología comparada, paleontología y sistemática para aproximarse a temas inabordable por la Biología Evolutiva tradicional. Nosotros hemos empleado esta aproximación integrativa para investigar el origen de innovaciones en la

pata de las aves vivientes. Utilizando datos y métodos filogenéticos pudimos identificar aspectos que demandaban una investigación embriológica, así como recíprocamente pudimos apoyar la interpretación de los datos embriológicos en los patrones morfológicos indicados por la filogenia.

El estudio de la condrogénesis tardía en relación a temas macroevolutivos comúnmente involucra la investigación de influencias recíprocas entre diferentes elementos del esqueleto (por ejemplo, en el desarrollo del caparazón de las tortugas, arriba mencionado). Estas interacciones tienen el potencial de, a partir de un cambio inicial en una estructura, generar modificaciones en otras estructuras. La importancia de las interacciones entre las partes del organismo durante el desarrollo es evidente en los casos de heterocronía durante la diferenciación del esqueleto (Meyer 1987, Miller 1991, Cubo et al. 2000, Weisbecker et al. 2008). Estructuras que se diferencian antes o después pueden originar modificaciones como alometrías (diferencias de tamaño entre estructuras) y paedomorfosis (retención de rasgos juveniles), teniendo gran impacto en la forma general del esqueleto. Otra clase de interacción importante durante la condrogénesis tardía es la acción muscular durante el desarrollo del esqueleto (Hall 1989, Wu et al. 2001, Blitz et al. 2009). La actividad muscular puede causar desde pequeños cambios de forma hasta modificaciones importantes, como la fusión, pérdida o aparición de nuevas estructuras. Estos dos mecanismos en sí – heterocronías e

influencias musculares – son reconocidamente importantes para el desarrollo y la evolución del esqueleto. En esta tesis mostramos además cómo estos dos procesos pueden estar relacionados en el origen de innovaciones evolutivas, al mostrar cómo retrasos en la diferenciación del esqueleto amplían la ventana temporal de influencia de la musculatura durante el desarrollo de los dígitos de las aves. El desarrollo del halux oponible es causado por la torsión del Mt1, el que a su vez es posibilitado por un retraso en la diferenciación de su cartílago. La evolución de reorientaciones secundarias del pie anisodáctilo es causado por reducciones de la musculatura del pie y es posibilitado por heterocronías en el desarrollo del esqueleto asociadas a la altricialidad.

Este tipo de investigación sobre mecanismos ontogenéticos responsables por innovaciones evolutivas contribuye también a la discusión general sobre los mecanismos evolutivos. La Biología Evolutiva hegemónica, inspirada en los modelos de la Teoría Sintética, considera variaciones en las frecuencias génicas en respuesta a demandas ecológicas como el agente determinante de la dirección de los cambios evolutivos. Este modelo, concebido antes del advenimiento de la genética molecular, presume una relación simplista entre la acción génica y la morfología. Esta simplificación no sólo hace que el modelo sea incompleto, sino que también lo hace impreciso, por ignorar cómo la construcción del fenotipo actúa direccionando el cambio evolutivo. En los casos estudiados en esta tesis, pudimos apreciar cómo las

transformaciones en la morfología de la pata de las aves son limitadas y frecuentemente convergentes. Las interacciones en el desarrollo del sistema musculoesquelético en conjunto con variaciones en la temporalidad del desarrollo han permitido generar la diversidad de fenotipos en la pata que hoy podemos observar asociada a una diversidad semejante en modos de vida.

Si la Biología Evolutiva del Desarrollo puede hacer un cambio significativo en la estructura de la teoría evolutiva, como actualmente es discutido por diversos autores, ésta debe expandir sus prácticas y métodos, y abarcar los múltiples niveles de la organización biológica. Esta tesis hace una pequeña contribución en este sentido al mostrar, desde un abordaje integrativo, cómo mecanismos ontogénicos son capaces de producir mucha de la diversidad del pie de las aves.



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Anexo 1

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## The developmental origin of zygodactyl feet and its possible loss in the evolution of Passeriformes

João Francisco Botelho, Daniel Smith-Paredes, Daniel Núñez-Leon, Sergio Soto-Acuña and Alexander O. Vargas

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# The developmental origin of zygodactyl feet and its possible loss in the evolution of Passeriformes

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The zygodactyl orientation of toes (digits II and III pointing forwards, digits I and IV pointing backwards) evolved independently in different extant bird taxa. To understand the origin of this trait in modern birds, we investigated the development of the zygodactyl foot of the budgerigar (Psittaciformes). We compared its muscular development with that of the anisodactyl quail (Galliformes) and show that while the musculus abductor digiti IV (ABDIV) becomes strongly developed at HH36 in both species, the musculus extensor brevis digiti IV (EBDIV) degenerates and almost disappears only in the budgerigar. The asymmetric action of those muscles early in the development of the budgerigar foot causes retroversion of digit IV (dIV). Paralysed budgerigar embryos do not revert dIV and are anisodactyl. Both molecular phylogenetic analysis and palaeontological information suggest that the ancestor of passerines could have been zygodactyl. We followed the development of the zebra finch (Passeriformes) foot muscles and found that in this species, both the primordia of the ABDIV and of the EBDIV fail to develop. These data suggest that loss of asymmetric forces of muscular activity exerted on dIV, caused by the absence of the ABDIV, could have resulted in secondary anisodactyly in Passeriformes.

## 1. Introduction

Differences in the morphology of the foot are among the main factors that allowed the specialization of the avian leg (for a review, see [1]). The ancestral condition to extant birds was a four-toed cursorial foot (but functionally tridactyl), as observed in non-avian theropods [2,3]. The anisodactyl foot—where the digits II, III and IV are oriented forwards, while digit I is oriented backwards—evolved in early Avialae by the retroversion of the hallux [4,5]. In extant birds, modifications of the anisodactyl foot produced similar morphologies independently in different taxa. Several families reduced or lost the hallux, among them some palaeognathous (ostrich and allies), Rallidae (rails), Mirandornithes (flamingos and grebes) and Charadrii (plovers, sandpipers and allies). Palmated feet with webs between the digits evolved in many species of aquatic birds. Further anatomical variation was generated by changes in the orientation of the digits. Zygodactyl foot have digits I and IV oriented backwards and digits II and III oriented forwards, and have evolved independently by the backward orientation of digit IV (dIV) in at least three extant clades: Cuculidae (cuckoos), Psittaciformes (parrots) and Piciformes (woodpeckers and allies). Some birds are semi-zygodactyl and can facultatively change the orientation of dIV, like Musophagidae (turacos), Pandionidae (ospreys), Strigiformes (owls) and Coliidae (mousebirds). An arrangement that is functionally similar to zygodactyly, named heterodactyly, has evolved in Trogoniformes (trogons), where digits I and II are oriented backwards, and digits III and IV are oriented forwards. Finally, swifts (Apodidae) have a pamprodactyl foot, a condition where all four digits can be oriented forwards.

It is remarkable that almost every variation of the anisodactyl foot has evolved independently in different lineages. This suggests that not only ecological demands, but also developmental factors could have driven the direction of evolutionary changes [6,7]. To understand the nature of those factors, we explored one of these transformations—the origin of the zygodactyl feet—from a developmental perspective.

Previous studies about the origin of zygodactyly have focused on its functionality and on its underlying musculoskeletal organization. Zygodactyly has often been considered as an adaptation for climbing, perching or manipulation [1,8–10]. However, taking into account the diversity of niches occupied by extant zygodactyl species, and also the fact that those same niches are occupied by non-zygodactyl birds, it is not straightforward to find a common evolutionary scenario to explain its origin. Moreover, anatomical comparisons show that there is not a common muscular anatomy to all zygodactyl feet [11–14], making it difficult to recognize which muscular topology is specifically related to that arrangement. The digits are controlled by a complex combination of individual muscles. Flexor muscles, responsible for grasping movements, are found in the ventral side, while extensor muscles, responsible for releasing, are found in the dorsal side. Adductor and abductor muscles are defined in relation to the limb axis. Therefore, the abductor of dIV is the muscle inserted on the lateral side of dIV, while the abductor of digit II (dII) is inserted on the medial side of dII. The muscles that control the digits can be further classified according to their location into intrinsic and extrinsic muscles. The extrinsic muscles are situated in the crus, while the intrinsic muscles are situated in the foot. Each zygodactyl taxon differs in the number and the organization of those muscles.

In this work, we investigate the development of the foot musculoskeletal system of a zygodactyl bird—the budgerigar (*Melopsittacus undulatus*)—to discern which factors are causally related to its development and to understand the possible causes of its convergent evolution. For appropriate inference, we also examined the development of the foot of the Japanese quail (*Coturnix japonica*) and zebra finch (*Taeniopygia guttata*), two anisodactyl birds with radically different evolutionary histories.

## 2. Material and methods

### (a) Animals

Fertilized eggs of Japanese quail (*C. japonica*), zebra finch (*T. guttata*) and budgerigar (*M. undulatus*) were obtained from colonies at the University of Chile. The eggs were removed from nests and incubated in an incubator with automatic rotating shelves at 37.5°C and 60% humidity. The nomenclature employed for the skeleton, muscles and tendons follow *Nomina Anatomica Avium* [15]. The embryos were staged using the normal table for *Gallus gallus* [16].

### (b) Cartilage and bone stain

Developmental series for each species investigated were prepared for skeletal staining. Embryos were fixed in 100% methanol for at least 2 days. Cartilage was stained with a solution of 0.02% Alcian Blue (Sigma-Aldrich) diluted in 5:1 ethanol/acetic acid for 24–48 h. Bone was stained with a solution of 0.02% Alizarin Red (Sigma-Aldrich) diluted in water with 0.5% of KOH for 2 h. The excess of dye was washed with water, and the muscles were macerated with 2% KOH. The embryos were cleared in a series of glycerol.

### (c) Immunohistochemistry and immunofluorescence

Four embryos for each stage were used for immunofluorescence against myosin (*C. japonica*, *M. undulatus* and *T. guttata*) and tenascin (*M. undulatus*). Embryos were fixed in Dent's fix (4:1 methanol:DMSO) for 2 h, dehydrated in a series of methanol and left for at least 12 h at  $-80^{\circ}\text{C}$ . Then, the specimens were bleached in Dent's bleaching (4:1:1 methanol:DMSO:H<sub>2</sub>O<sub>2</sub>) for 24 h at room temperature. They were rehydrated in phosphate buffer with 1% triton X-100 (Sigma) (PBST). Primary antibodies against myosin (MF-20 from DSHB, Iowa) and tenascin (M1-B4 from DSHB, Iowa) were diluted 1:20 in PBST, 5% horse serum and 5% DMSO. Embryos were kept in primary antibody for 48 h at 4°C in an orbital shaker. Embryos were washed in PBST six times for 1 h. Secondary antibodies anti-mouse made in donkey coupled to horseradish peroxidase (715-035-150, Jackson ImmunoResearch) or Alexa-Fluor 594 (715-585-150, Jackson ImmunoResearch) were diluted 1:300 in PBST, 2% horse serum and 5% DMSO. Embryos were kept in secondary antibody for 24 h at 4°C in an orbital shaker. They were washed again in PBST six times for 1 h. Embryos labelled with horseradish peroxidase were revealed with Diaminobenzidine substrate (11718096001, Roche Applied Bioscience). To avoid background, the first 10 min of reactions were carried out in ice.

### (d) Paralysis

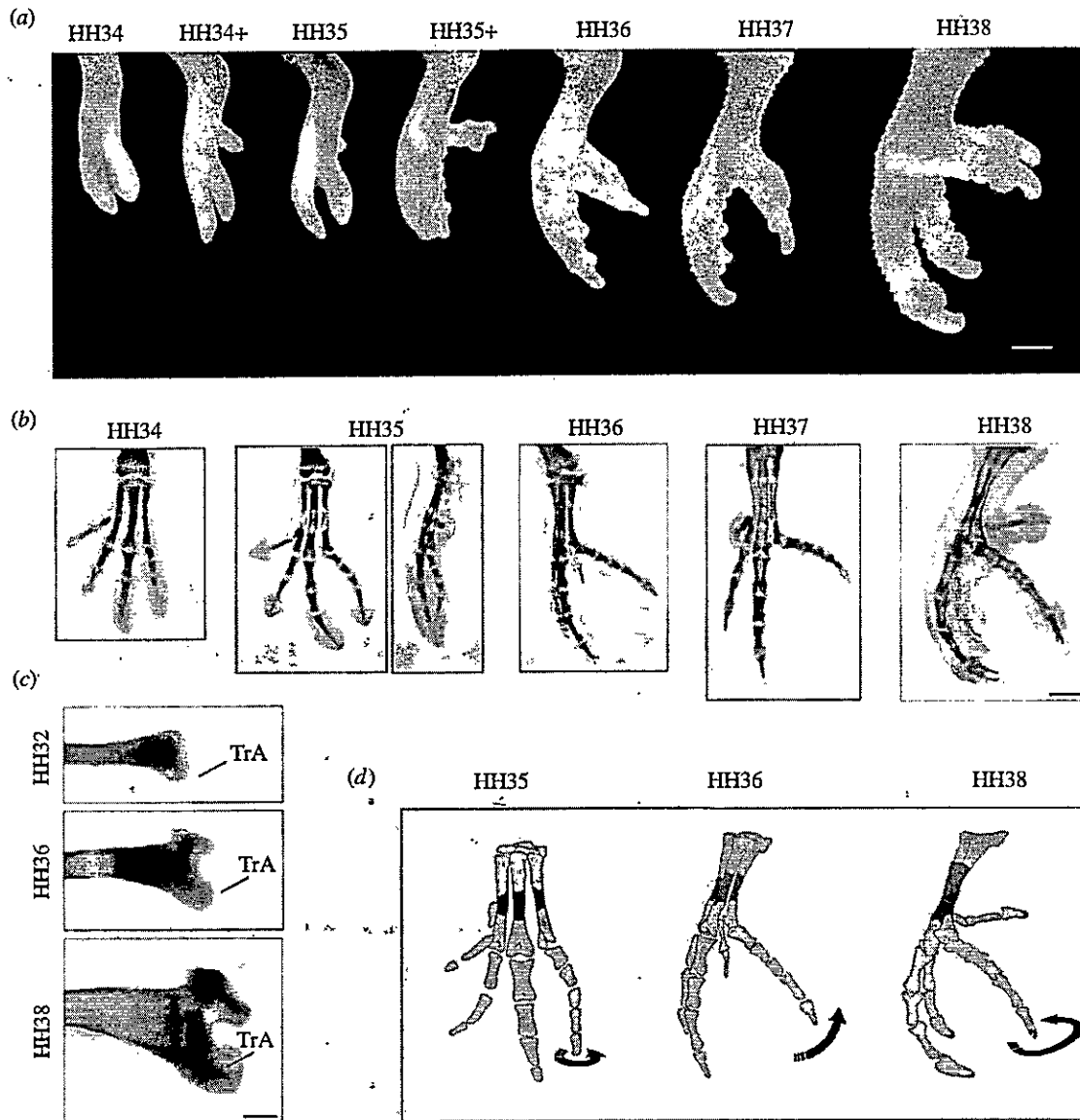
Fourteen budgerigar embryos were pharmacologically paralysed; eight survived. After candling the egg, a small hole was opened with a needle over the air sac. A single dose of 20  $\mu\text{l}$  of a solution containing 2 mg ml<sup>-1</sup> of decamethonium bromide diluted in phosphate (modified from [17]) was then delivered with a micropipette to embryos at HH31 [16]. The egg was sealed with a glue gun and incubated without movement for 4 days.

## 3. Results

### (a) Rotation of digit IV in budgerigar development (Psittaciformes)

The first step to understand the development of the zygodactyl foot was to determine when and how dIV changes its orientation, departing from the development of the anisodactyl foot. Limb development is well known in chicken and quail, and thus these animals yield good anisodactyl models to compare with the budgerigar. In these taxa, the toes develop from cartilages originated in a pad-like flattening of the distal limb bud. Chicken limbs exhibit the cartilages of the four digits at HH31, except for the distal-most phalanges [16]. At this stage, toes are united by mesenchymal tissue and oriented in the same plane. Posteriorly, the tissue between the toes becomes a thin web, the digits elongate conspicuously, and a collar of bone begins to grow at the centre of each metatarsus (HH34). Eventually, the digits separate from each other (HH35), the hallux rotates ventrally and the primordia of the claws appear (HH36).

We produced a developmental series for the budgerigar stained with Alcian Blue and Alizarin Red. At early stages, the development of the foot of the budgerigar does not differ from what has been described for the domestic chicken. The first sign of zygodactyly occurs around HH35, when dIV rotates medio-laterally at the level of the articulation between the metatarsus and the proximal phalanx (figure 1a,b). Consequently, the medial side of the phalanges of dIV comes to face dorsal and the lateral side to face ventral. The dIV is then flexed and acquires a right angle in relation to the main axis of the



**Figure 1.** (a) The anatomy of budgerigar early foot development. (b) The anatomy of budgerigar early hindlimb skeletal development. Cartilages stained with Alcian Blue, bones stained with Alizarin Red. (c) The trochlea accessoria (TrA) at different stages: MtIV has been isolated and photographed in lateral view. (d) Diagram picturing the change of dIV orientation during development. Scale bars: (a,b) 1 mm; (c) 200  $\mu$ m. (Online version in colour.)

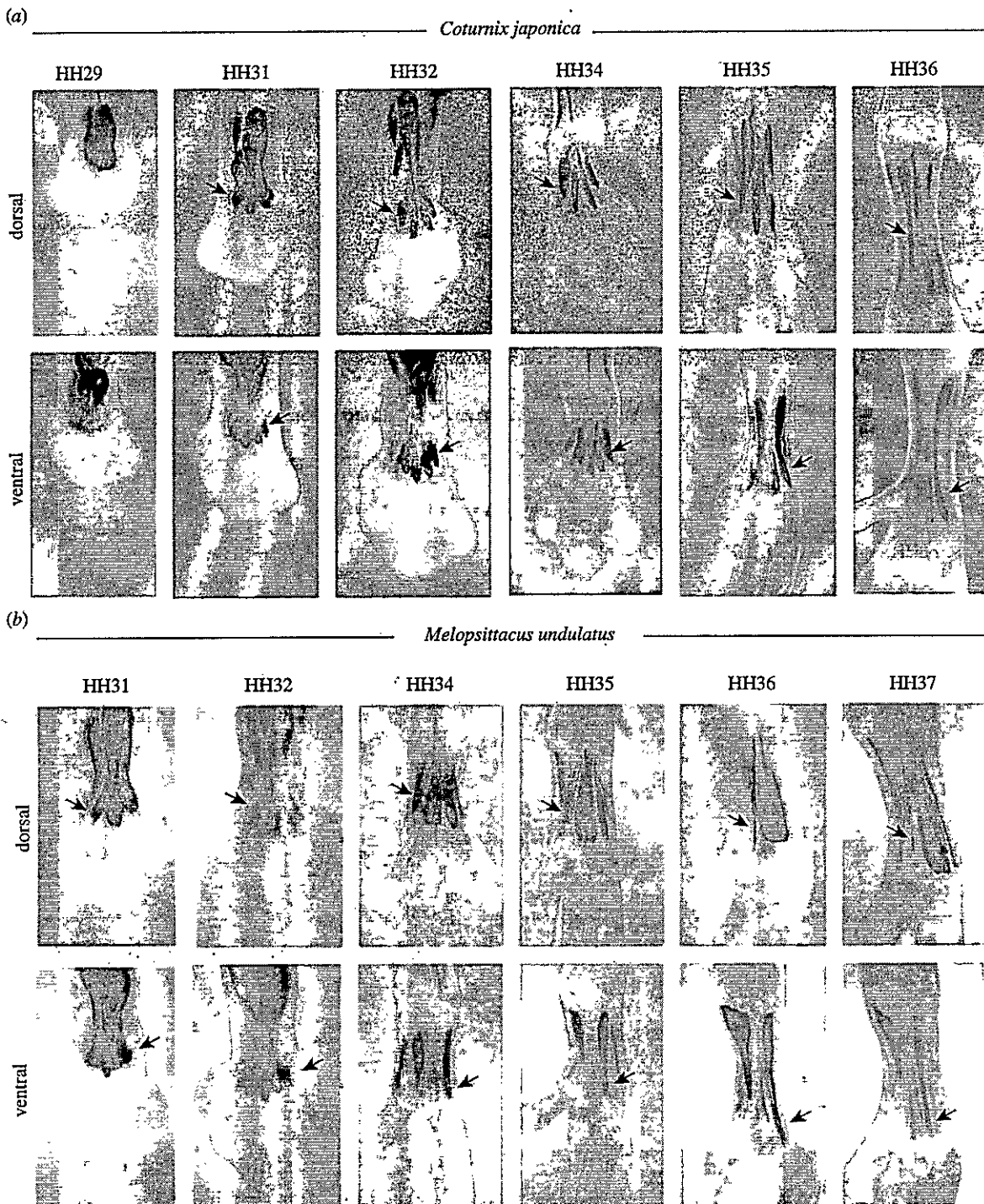
metatarsus (HH36). The morphology at this stage is similar to that of adult semi-zygodactyl birds like owls and touracos. Eventually, dIV rotates further towards medial and the foot becomes fully zygodactyl (HH37). The rotation and flexion of dIV is followed by a ventral outgrowth of the distal end of metatarsal IV. First, it develops a wing-like flange similar to the trochlea accessoria (TrA) observed in semi-zygodactyl birds (HH36); later it acquires the hooked shape (or *sehnehalter*) characteristic of the TrA of fully zygodactyl birds like Psittaciformes and Piciformes (figure 1c). In summary, during the development of the budgerigar foot, dIV rotates at HH35, flexes at HH36 and further rotates at HH37 (figure 1d).

### (b) Extensor brevis digiti IV is lost in the development of the budgerigar

The time and mode of change in orientation of dIV suggest that early muscular activity could be acting in the transformation of the foot. The early development of avian hindlimb muscles has been studied in the domestic chicken [18–21].

Muscle cell precursors that originated in the lateral somite migrate to the limb bud around HH22 [22]. The precursor cells generate a dorsal and a ventral mass of differentiating muscle fibres. Each mass divides, generating successively smaller masses, and eventually forming each individual muscle around HH35. Movements of the ankle and digits begin at HH32 and at HH35, respectively [23].

The complex combination of muscles and tendons controlling bird toes is highly variable among taxa. There is a large amount of literature concerning this variation, as it has been employed to investigate the high-order phylogeny of birds [12,24,25]. Those studies suggest that for Neornithes, the ancestral condition for the muscles controlling dIV is similar to the condition observed in Galliformes, like the chicken and the quail. In those birds, the extension of dIV is controlled by one extrinsic muscle attached to the dorsal side of the distal phalanx (*M. extensor digitorum longus*) and one intrinsic muscle attached to the medial side of the proximal phalanx (*M. extensor brevis digiti IV*—EBDIV). The medial insertion of EBDIV probably enables it to act



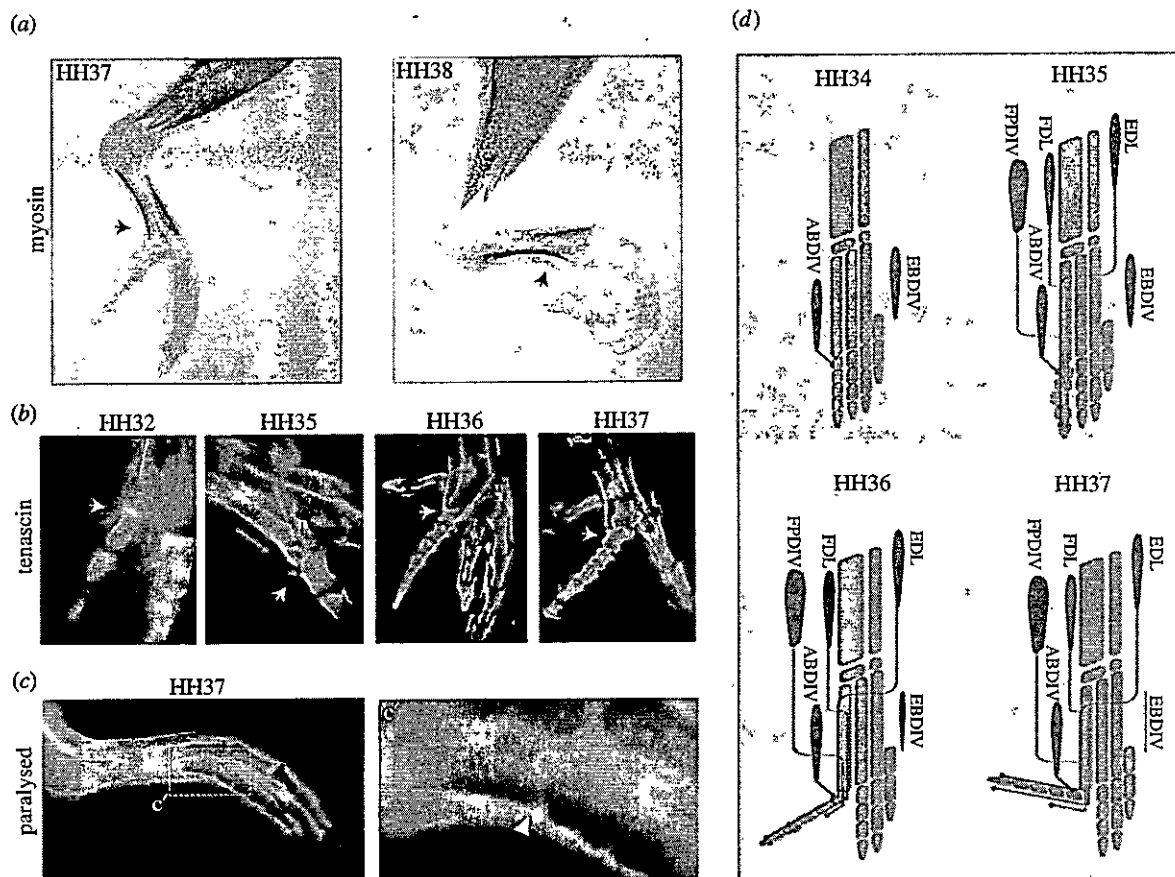
**Figure 2.** Immunohistochemical reaction against myosin 2 showing the early development of dorsal and ventral foot muscles in (a) quails and (b) budgerigars. Black arrows indicate the EBDIV. (Online version in colour.)

also as an adductor. The flexion of dIV is controlled by two extrinsic muscles attached to the distal phalanx (*M. flexor digitorum longus* and *flexor perforatus digiti IV*), while its abduction is produced by a ventral intrinsic muscle inserted in the lateral face of the proximal phalanx (*M. abductor digiti IV—ABDIV*) (figure 3*d*).

To compare the development of the muscles controlling dIV in the anisodactyl quail and the zygodactyl budgerigar, we examined embryos immunostained for myosin type 2. We found that the development of the extrinsic muscles is very similar in both species. However, the two species differ in the intricate set of fusions, changes in size and displacement of

the muscle belly in relation to the metatarsus that are undergone by the intrinsic muscles.

Initially, the feet of both species exhibit the primordia of four dorsal and four ventral muscles (figure 2*a,b*). The most lateral primordia of the dorsal and ventral sides of the foot generate the EBDIV and ABDIV, respectively. Both muscles become well developed by HH36 in quail embryos. Nevertheless, in budgerigar embryos, EBDIV degenerates soon after it separates from the other muscles and is reduced to a thread at HH37 (the presence of a small EBDIV has been reported for one species of parrot [13], but the muscle is absent in other described Psittaciformes [11,26]). On the



**Figure 3.** (a) Immunohistochemical reaction against myosin 2 showing the late disposition of the ABDIV (black arrows) in relation to changes in the orientation of dIV in budgerigars. (b) Immunohistochemical reaction against tenascin showing the insertion of the tendon of the ABDIV (white arrows) during budgerigar development. (c) Morphology of dIV in paralysed embryos; (c') detail of the trochlea accessoria. (d) Diagram illustrating the development of the musculoskeletal system in the budgerigar. FDL, flexor digitorum longus; EDL, extensor digitorum longus; FPDIV, flexor perforatus digiti IV. (Online version in colour. In (d), muscles are shown in red, skeleton in blue.)

other hand, the ABDIV becomes strongly developed and its muscle belly extends for four-fifths of the metatarsal length (figure 3a).

These differences in muscle size could generate an asymmetric relation of forces, which is coherent with the rotation of dIV observed at HH35. The absence of a muscle inserted in the medial side and a stout muscle in the lateral side could account for the rotation of dIV from medial to lateral. Then, as the lateral side of the phalanges passes to face ventral, the action of ABDIV results in flexion of the digit, which acquires a right angle in relation to the limb main axis (HH37; figure 3b,d). Further flexion of dIV by action of ABDIV, while being constrained by the extensor muscles, would lead to the final zygodactyl orientation, attained at HH38.

### (c) Muscular paralysis in budgerigar results in anisodactyl feet

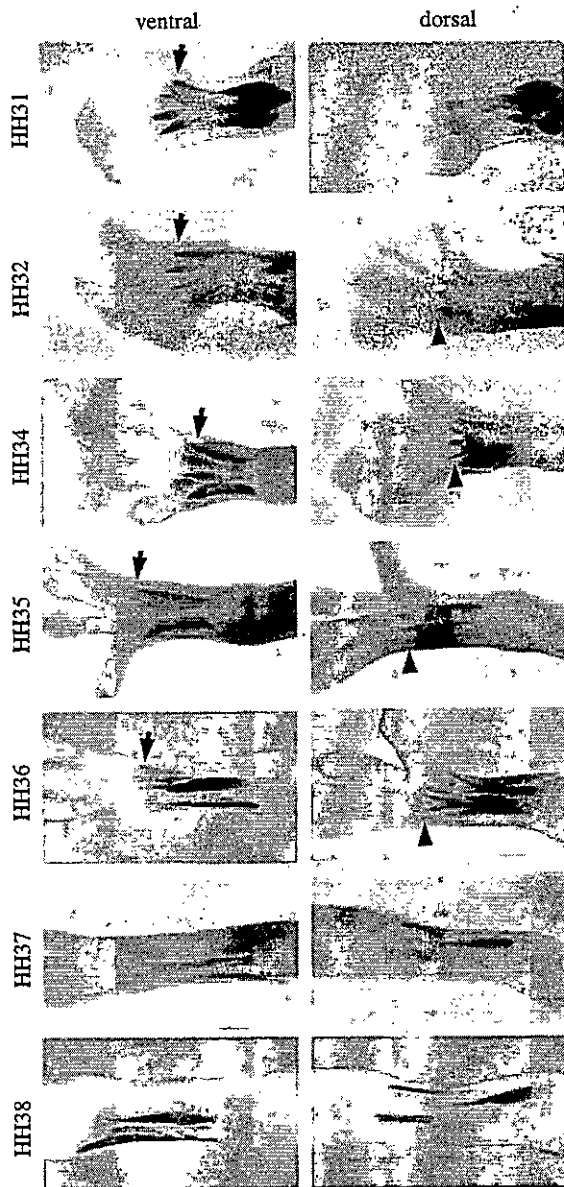
To test the hypothesis of the influence of early muscular activity on the development of zygodactyly, we pharmacologically immobilized budgerigar embryos and examined the resulting foot phenotype. The *in ovo* injection of the cholinergic agonist and neuromuscular blocker decamethonium bromide ( $n = 8$ ) produced paralysis in budgerigar embryos. Treated embryos were anisodactyl at HH37 and HH38, and did not develop the TrA (figure 3c). Some other

typical deformities observed in paralysed chickens [27] were also observed in paralysed budgerigars, such as reduced body size, incomplete fusion of the sternum and non-fused mandibular symphysis. Nevertheless, paralysed budgerigars did not exhibit any major general deformities that could justify the lack of rotation of dIV.

### (d) Both extensor brevis digiti IV and abductor digiti IV are lost in the development of the zebra finch (Passeriformes)

Most foot muscles are known to have been lost or vestigialized in Passeriformes [25,28], but their early development is still unknown. Taking into account the influence of muscle action on the development of zygodactyly in the budgerigar, we investigated the development of hindlimb muscles in the anisodactyl foot of the zebra finch (*T. guttata*). We observed that, similar to quails and budgerigars, the primordia of four ventral and four dorsal muscles are present at early stages (figure 4). However, most intrinsic muscles become vestigial during development and are almost indiscernible at HH37 (figure 4). The absence of both EBDIV and ABDIV acquires special significance since phylogenetic analyses of fossil and molecular data suggest that Passeriformes could have evolved from zygodactyl ancestors, and are thus secondarily anisodactyl (see Discussion).





**Figure 4.** Immunohistochemical reaction against myosin 2 showing the early development of dorsal and ventral foot muscles in zebra finch. Most muscles disappear at HH37. Arrows, EBDIV; arrow heads, ABDIV. (Online version in colour.)

#### 4. Discussion

Whole-mount immunostaining of the small developing muscles and tendons allowed us to compare the embryonic musculoskeletal system of anisodactyl and zygodactyl birds. These observations revealed that changes in the orientation of budgerigar dIV during ontogenesis occur concomitant to the modifications undergone by the intrinsic muscles controlling it. While the anisodactyl quail develops fully functional ABDIV and EBDIV, the zygodactyl budgerigar fails to develop an EBDIV. In the absence of the EBDIV restricting dIV movements, the abduction of dIV could cause the ontogenetic transition from anisodactyly to zygodactyly. Consistently, paralysed budgerigar embryos develop an anisodactyl foot, which confirms that muscle activity is necessary for the ontogenetic reorientation of dIV. In summary, our new data provide compelling evidence that the development of the zygodactyl foot in the budgerigar is caused by the asymmetric action of intrinsic muscles controlling dIV.

As the strong extrinsic flexor and extensor muscles can control more than one digit, most studies of the zygodactyl foot have looked for a common muscular organization of the extrinsic muscles controlling both retroverted digits dI and dIV in zygodactyl birds [1,8]. Our investigation of the ontogenesis of the musculoskeletal system identifies the reduction of small intrinsic muscles simultaneous to digit retroversion as the most likely cause for the development of zygodactyly in the budgerigar. The identification of the importance of intrinsic muscles brings an important new aspect to consider in the evolution of specializations in the avian foot.

#### (a) Epigenesis and convergence

The development of the musculoskeletal system depends on the functional interlocking of initially independent processes. Muscles, tendons and bones have different embryological origins. When brought together, their interactions have reciprocal morphogenetic effects [29]. Consequently, those interactions are potential sources of variation. Modifications in the early muscle precursor tissue can cause variations in the skeleton, and vice versa. The developmental mechanism here proposed for the origin of zygodactyly provides an example of the power of those embryonic interactions to generate and drive evolutionary transformations [7,30].

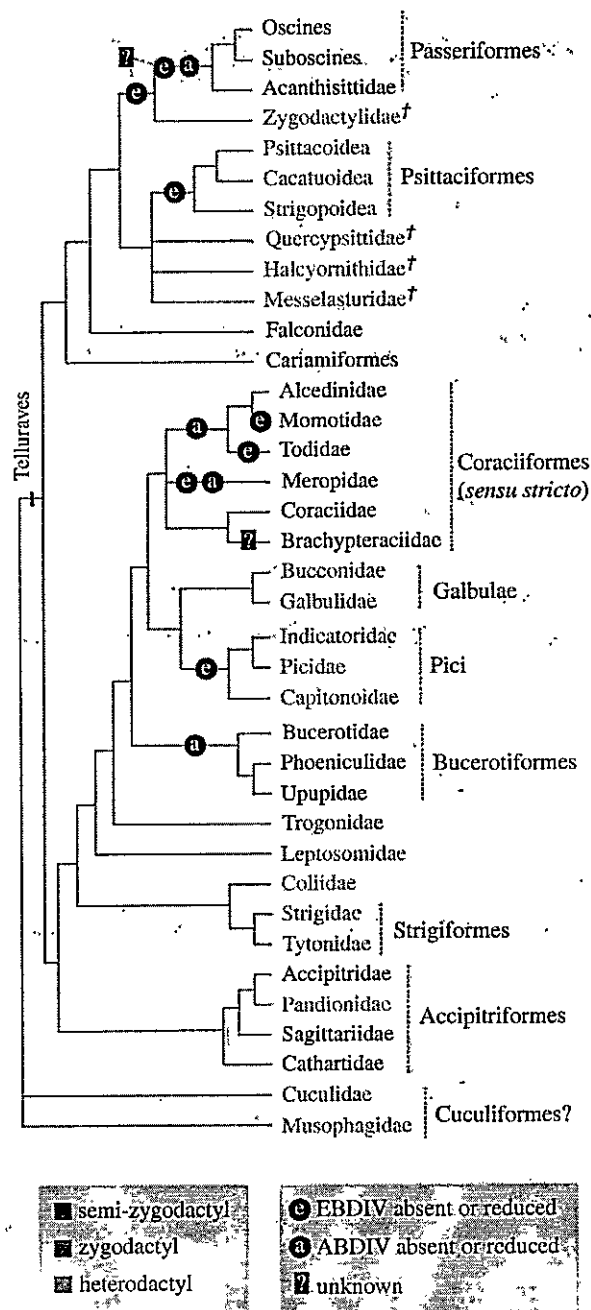
The avian tarsometatarsus originates from the fusion among the diaphyses of metatarsals II, III and IV, and the distal tarsal cartilage [31]. This fusion initially produces a common ossified diaphysis with three independent cartilaginous epiphyses in each side. At the proximal end, the interaction with tendons models the hypotarsus. At the distal end, the complex combination of muscles controlling the digits results in an intricate set of epigenetic influences over each of the individual epiphyses. For example, birds that lack intrinsic muscles of the foot usually present small incisuras intertrochlearis, narrow and parallel trochleae, etc. We propose that zygodactyly and the associated TrA are produced by this same kind of process. They result from the epigenetic influence of muscular forces over the skeleton.

The reoccurrence of a similar set of influences over the skeleton of related lineages could cause convergent evolution [32–34]. The early presence of the primordia of intrinsic muscles of the foot and its variable posterior disappearance or reduction yield a drive for the repetition of similar influences and, consequently, for the transformation of the avian foot skeleton. These two factors—the effect of muscle over the form of digits *plus* the bias for the transformation of intrinsic muscles—provide a mechanistic explanation for the convergent evolution of zygodactyly. The convergence would have been facilitated by the flexibility of the muscular system added to its influence over the skeleton. As muscles were reduced, lost or reacquired, similar skeletal morphologies evolved in parallel, including zygodactyly.

#### (b) Zygodactyly in extant and fossil birds

Zygodactyly has originated at least three times in extant birds: in Piciformes (Pici + Galbulae), Psittaciformes and Cuculidae (figure 5). The cuckoo-roller (*Leptosomus discolor*) may represent a fourth independent origin [41–43]. The developmental mechanism proposed for the origin of zygodactyly—an imbalance of forces in the lateral and medial sides of the proximal phalanx—may also explain the evolution of zygodactyly in Pici (barbets, toucans, woodpeckers and allies). They are the only other Telluraves (clade comprising most arboreal





**Figure 5.** Phylogeny of Telluraves plus Cuculidae and Musophagidae, depicting the position of Zygodactylidae and the loss of the intrinsic muscles of dIV. Clades depicted were congruently obtained by the earlier studies [35–37]. The position of Zygodactylidae and stem Psittaciformes follow [38–40]. (Online version in colour.)

birds)—besides parrots—that have lost EBDIV but conserve ABDIV (figure 5) [14,15].

Extant Galbulae [12] and Cuculidae [14,44,45] exhibit the EBDIV, and a furrow (sulcus extensorius) on the dorsal tarsometatarsus indicates that EBDIV is also present in stem Cuculidae [46,47], stem Psittaciformes [38,48–50] and stem Pici [10,51]. Those taxa have two characteristics in common: a TrA smaller than the one present in crown Psittaciformes and crown Pici [10,41,46–48,51,52], and an unusual trajectory of the EBDIV tendon: it does not pass through a foramen between mtIII and mtIV—the canalis interosseus distalis—as in most anisodactyl birds; it goes over the incisura intertrochlearis lateralis and backwards to its insertion on the medial side of the proximal phalanx (figure 6) [11,47,50,51,53].

If a mechanism similar to the one proposed here for crown Psittaciformes and crown Pici is responsible for the development of zygodactyly in other taxa, an imbalance of forces acting on the proximal phalanx of dIV may be caused by subtler dissimilarities, like differences in the size of the muscles or differences in the time and position of insertion of the tendons, as suggested by the unusual trajectory of the EBDIV tendon over the incisura intertrochlearis lateralis (not through the canalis interosseus distalis).

The fossil record also shows that the condition observed in crown Psittaciformes and crown Pici (absence of the EBDIV, and large TrA) is convergently derived from zygodactyl ancestors having a smaller TrA and the tendon of EBDIV passing over the incisura intertrochlearis distalis. This morphological pattern suggests that the form and size of the TrA are related to the degree of imbalance of the forces acting over the metatarsus during development: those birds who lost or vestigialized EBDIV—like crown Pici, crown Psittaciformes and, probably, Zygodactylidae (see below)—exhibit larger TrA than those taxa that keep an EBDIV, like Galbulae and Cuculidae.

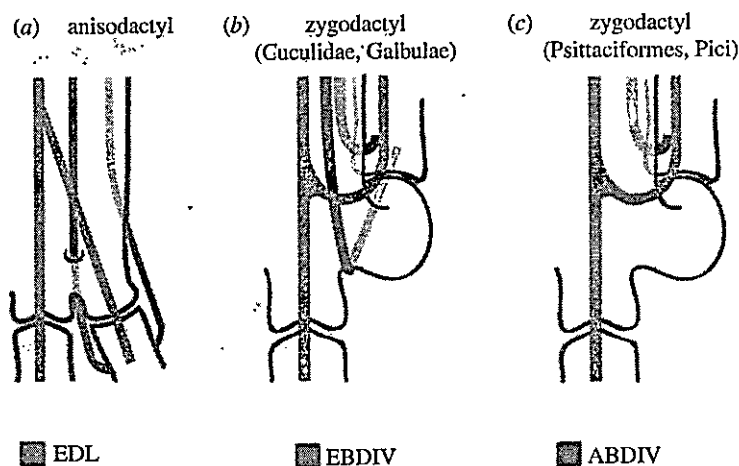
### (c) Zygodactylidae and the loss of abductor digiti IV in Passeriformes

The presence of a TrA in the distal metatarsal of dIV allows the identification of zygodactyly even in non-articulated fossil skeletons. A group of those fossils has been recognized as a family of extinct birds appropriately called Zygodactylidae [10,39]. Even though they exhibit a well-developed TrA in the metatarsus IV, similar to that found in Pici and Psittaciformes, cladistic analysis suggests that Zygodactylidae is the sister taxon of passerines (Passeriformes) (figure 5) [40]. On the other hand, one of the most robust data produced by the new avian molecular phylogenies is the sister relationship between Passeriformes and Psittaciformes [35–37,54–57]. Therefore, both the extinct and extant outgroups to Passeriformes are zygodactyl, suggesting that Passeriformes had zygodactyl ancestors [10].

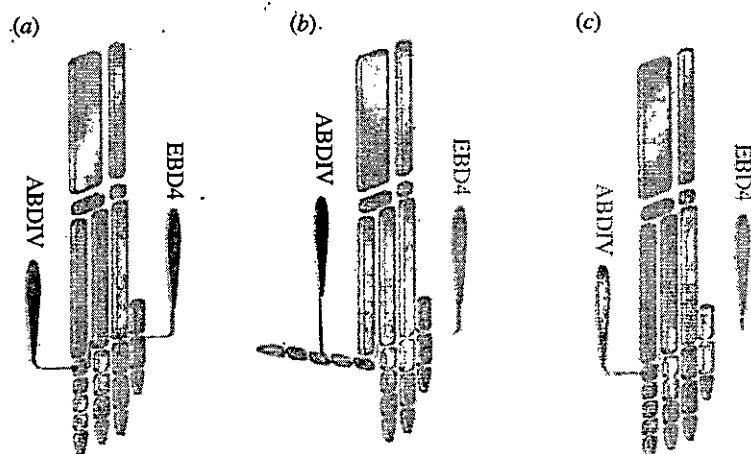
Considering our hypothesis on the role of ABDIV in the re-orientation of dIV in Psittaciformes, this muscle would be expected to have been present in Zygodactylidae. Therefore, the inferred ancestral state for the hypothetical clade (Psittaciformes (Zygodactylidae + Passeriformes)) would be the presence of ABDIV (figure 5). In this scenario, the further reduction of ABDIV in the lineage of passerine birds could have caused the loss of zygodactyly, making them secondarily anisodactyl. Passeriformes would be anisodactyl not by an equilibrium of forces, as in primarily anisodactyl birds, but by the absence of any force acting on the lateral and medial sides of the proximal phalanx (figure 7), similar to paralysed budgerigars. The presence of the primordia of the intrinsic muscles of the foot and their differential loss during the development of each taxon indicate the phylogenetic flexibility of the trait and support this possibility.

### (d) Heterodactyly

The singular arrangement known as heterodactyly is unique to birds of the family Trogonidae. Those birds have digits I and II reverted, and digits III and IV pointing forwards. Molecular phylogenies include trogons in the Telluraves assemblage [35], but its specific position is unresolved. If a similar mechanism of asymmetrical forces at early development is responsible for the unique change of digit II orientation found in trogons,



**Figure 6.** The topology of div tendons. (a) Anisodactyl, (b) zygodactyl with EBDIV and (c) zygodactyl lacking the EBDIV. In (b), the EBDIV does not pass through the *canalis interosseus distalis*. In (c), the tendon is absent. Modified from [11]. (Online version in colour.)



**Figure 7.** The distribution of div intrinsic muscles in (a) the primary anisodactyl quail, (b) the zygodactyl budgerigar and (c) the putative secondary anisodactyl zebra finch. Budgerigar lost the EBDIV but conserves the ABDIV. Both muscles are absent in the zebra finch. (Online version in colour. Muscles are shown in red, skeleton in blue.)

the loss of muscles ought to have occurred in the opposite sides to that found in zygodactyl birds. The dorsal musculus abductor digiti II (ABDII) is inserted in the medial side of proximal phalange of the dII and the ventral musculus adductor digiti II (ADDII) is inserted in the lateral side. As the toe must rotate in the opposite direction in relation to the zygodactyl feet, the rotation would demand the loss of the ventral intrinsic muscle (ADDII). The hindlimb muscles of two species of the genus *Trogon* have been described by Maurer & Raikow [58]. The species described present the ABDII but do not have the ADDII, suggesting that a similar mechanism of asymmetric muscular action could be responsible for the development of heterodactyly. Furthermore, they are the only Telluraves clade that have lost ADDII but not ABDII, since other clades that do not have ADDII also lost ABDII (some Coraciiformes, Upupiformes, Pici, Psittaciformes and Passeriformes) [12,28,58].

## 5. Conclusion

Extant birds exhibit a diversity of foot forms, which is absent in any other archosaur group. The mechanism here proposed for the origin of zygodactyly is based on transformations that indirectly allowed that diversification in Aves. While the intrinsic muscles of the foot are relatively small and homogeneous

in crocodylians and theropod dinosaurs, birds have larger, more variable and individualized muscles [59,60], which allowed their differential influence on the skeleton. Furthermore, zygodactyl birds are very altricial and maintain the skeleton cartilaginous for most of the embryonic period. The action of the musculature on the avian embryonic skeleton has been recognized by different authors as an evolutionary mechanism [27,61,62]. We propose that foot muscle diversity and their action over the flexible embryonic skeleton caused the appearance of new foot configurations in birds, like zygodactyly. New fossil discoveries and further investigations on the anatomy and, especially, the development of the foot of other zygodactyl birds will contribute to test this hypothesis and further clarify the relationship between foot morphology and muscle development.

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